POLSKIE TOWARZYSTWO MIKROBIOLOGÓW
POLISH SOCIETY OF MICROBIOLOGISTS

Polish Journal of Microbiology

2018

Polish Journal of Microbiology
formerly Acta Microbiologica Polonica
2018, Vol. 67, No 3

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Purchase of DOI numbers for articles and implementation
of a modern publication platform
for the Polish Journal of Microbiology are funded with the financial support
of the Ministry of Science and Higher Education
under agreement No. 659/P-DUN/2018 for science dissemination activities.

Polish Journal of Microbiology
2018, Vol. 67, No 3, 251–258
https://doi.org/10.21307/pjm-2018-044

MINIREVIEW

Are Probiotic Really Safe for Humans?

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Submitted 29 March 2018, revised 6 May 2018, accepted 14 June 2018

Abstract

Probiotic bacteria have been used as a health-promoting factor for a very long time. Nowadays, products containing probiotic bacteria are becoming more and more popular on the market. The term probiotics refers to the products belonging to the following groups: probiotic drugs (medicinal products – live biotherapeutic products for human use), medical devices, probiotic foods (e.g. foods, food ingredients, dietary supplements or food for special medical purposes), directly fed microorganisms (for animal use) and designer probiotics (genetically modified probiotics). Safety assessment of bacterial strains used as probiotics should be carefully studied. Even though probiotic bacteria have the generally recognized as safe (GRAS status), there are several reports about side effects triggered by the presence of these organisms. Microorganisms used as probiotics may cause systemic infections, stimulate the immune system, disturb metabolism and participate in horizontal gene transfer.

Key words: bacteremia, gene transfer, probiotic bacteria, probiotic side effect, safety of probiotics

Introduction

The probiotics are live microorganisms that, when administrated in adequate doses, confer a health benefit to the host as defined by the Food and Agriculture Organization of the United Nations and the World Health Organization (FAO/WHO 2001). For this purpose, lactic acid bacteria (LAB) have been used for a very long time. At the beginning of the twentieth century, Ilja Metchnikoff suggested that the longevity of Bulgarians is due to consumption of fermented milk products (Metchnikoff 1908). Another scientist, Henry Tissier observed that in stools from children with diarrhea, only a small number of bacteria with Y-shaped morphology was present, while in healthy children, a large amount was observed (Tissier 1906). In 1965, Lilly and Stillwell used term "probiotic" to describe substances that were secreted by one organism and stimulated the growth of another (Lilly and Stillwell 1965).

Probiotic properties have been observed in many genera of bacteria and fungi, but the most commonly used probiotics belong to the species of *Lactobacillus* and *Bifidobacterium*. In addition, other bacteria genera, like *Streptococcus*, *Enterococcus*, and *Bacillus*, as well as members of the yeast genus *Saccharomyces* can have probiotic properties (Hempel et al. 2011). The most common species include: *Lactobacillus acidophilus*, *Lactobacillus johnsonii*, *Lactobacillus gasseri*, *Lactobacillus casei*, *Lactobacillus rhamnosus*, *Lactobacillus plantarum*, *Bifidobacterium longum*, *Bifidobacterium breve*, *Bifidobacterium bifidum* and *Bifidobacterium infantis* (Ishibashi and Yamazaki 2001). Some bacteria, not regularly present in the gastrointestinal tract, like *Lactobacillus bulgaricus*, *Streptococcus thermophilus*, *Leuconostoc* and *Lactococcus* species may also belong to the category of probiotic microorganisms and are usually used as starters in dairy products (Ishibashi and Yamazaki 2001). Most probiotic species, including lactobacilli, *Bifidobacterium*, lactococci, and some yeasts, are classified as the "generally recognized as safe" (GRAS). But there are groups of organisms, like streptococci, enterococci, *Bacillus* and other spore-forming bacteria, that do not possess GRAS status but have been used as probiotics (Snydman 2008). It should be noticed that not all bacteria of a given genus or species have probiotic features, they are assigned only to specific

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strains (Hill et al. 2014). The origin of the strain, resistance to antibiotics, as well as the lack of pathogenicity determines the safety of probiotic strains (Markowiak and Śliżewska 2017).

The term "probiotic" can refer to the following products: probiotic drugs (medicinal products – live biotherapeutics products for human use), medical devices, probiotic foods (e.g. foods, food ingredients, dietary supplement or food for special medical purposes), directly fed microorganisms (for animal use) and genetically modified probiotics (Venugopalan et al. 2010). Probiotics have an annual market growth of 7% globally (Johnson and Klaenhammer 2014; McFarland 2015) and are projected to grow to $65 billion in 2024 (Global Market Insights 2017). Guidance is required to assess the safety of probiotics, but several issues should be taken into account before, i.e., a large variety of probiotic strains, the risks associated with the use of unclassified strains, and the possibility of unsurpassed interactions between both the strains used and the host and bacteria (Gueimonde et al. 2004). Probiotics may be responsible for systemic infections (Marteau 2001; Doron and Snydman 2015), excessive immune stimulation in susceptible individuals (Marteau 2001; Borriello et al. 2003; Doron and Snydman 2015), deleterious metabolic effects (Marteau 2001; Doron and Snydman 2015) or gene transfer (Marteau 2001; Ashraf and Shah 2011; Doron and Snydman 2015). The factors that must be considered in assessing the safety of probiotic products should include infectivity, pathogenicity, an excessive immune stimulation in susceptible individuals, virulence factors comprising toxicity, metabolic activity and the important properties of microbes (Ishibashi and Yamazaki 2001). The Lactic Acid Bacteria Industrial Platform has submitted reports from the European Union indicating that except for enterococci the risk of infections caused by lactic acid bacteria is very low (Liong 2008).

Infections

The *Lactobacillus* strains are present in healthy humans in the oral cavity, ileum and colon; moreover, they are the main microorganisms in the vagina (Borrielo et al. 2003). The *Lactobacillus* and *Bifidobacterium* strains as commensals of human microbiome are safe and nonpathogenic. There is no evidence that consumption of probiotic lactobacilli or bifidobacterial strains poses the risk of infection greater than that related to commensal strains. The number of cases of infection caused by *Lactobacillus* or *Bifidobacterium* strains are very low and vary between 0.05–0.4% for infective endocarditis and bacteremia (Borrielo et al. 2003). The translocation of bacteria may be caused by a weakened intestinal barrier, resulting in the passage of bacteria across the mucus membrane and epithelium (Berg 1985). The next step is transport of microorganisms through the tunica propria to the mesenteric lymph nodes and other organs (Ishibashi et al. 2001). This translocation may result in bacteremia, followed by multiple organ failure and septicemia (Berg 1992; Van Leeuwen et al. 1994). Various factors such as damage of the mucous membrane, disturbances in the composition of the intestinal microflora, or diminishing of the host's immune system may stimulate the translocation of the intestinal bacteria (Ishibashi et al. 2001). In some clinical reports, probiotic bacteria have been identified as casual factors of dental cavities, endometritis (Bayer et al. 1978), urinary tract infections (Brumfitt et al. 1981; Dickgieber et al. 1984), meningitis (Sharpe et al. 1973) and spleen abscesses (Sherman et al. 1981). These infections can be associated with recent surgery, organ transplant, AIDS (Patel et al. 1994; Horwitch et al. 1995; Land et al. 2005; Ambesh et al. 2017), valvopathy, diabetes mellitus, immunosuppressive cancer therapy or cancer with antibiotic treatment, which may contribute to selection of specific microorganisms (Liong et al. 2008). Increased consumption of products containing probiotic bacteria has not led to an increase of the aforementioned opportunistic infections in consumers (Borrielo et al. 2003). In an epidemiological study of *Lactobacillus* bacteremia frequency in Finland, no correlation was found between the increased use of *L. rhamnosus* GG and the occurrence of *Lactobacillus* bacteremia during 1990–2000 (Salminen et al. 2002).

The cases of children infections, including bacteremia, caused by the *Lactobacillus* strains are quite rare and have been observed mainly in immunocompromised children (Bayer et al. 1978; Kalima et al. 1996; Schlegel et al. 1998, Land et al. 2005), and in patients with neonatal sepsis and meningitis (Brughton et al. 1983; Thompson et al. 2001), pneumonia (Sriskandan et al. 1993), and also local suppurative infections (Brook 1996). Probiotic therapy administered to preterm infants and neonates should be considered carefully, since at birth infants do not have a fully developed immune system, and thereby after probiotic administration the risk of fungemia or bacteremia significantly increases (Boyle et al. 2006; Marodi 2006). *Lactobacillus* bacteremia among children is very unusual, however, there were reports on three infants with short-bowel syndrome that developed bacteremia after consumption of *L. rhamnosus* GG (Kunz et al. 2004; De Groote et al. 2005). Land et al. (2005) reported two pediatric patients who had ingested probiotic strain *L. rhamnosus* GG and subsequently developed bacteremia and sepsis due to infection with *Lactobacillus* species. Vahabnezhad et al. (2013) described a case of *Lactobacillus* bacteremia in a 17-year-old boy with ulcerative colitis after ingestion

of *L. rhamnosus* GG. The 16S rRNA genes similarity was 99.78% between the *L. rhamnosus* strain isolated from the patient's blood and the consumed probiotic strain. This information suggests that people with ulcerative colitis are potentially at the risk of bacteremia due to the *Lactobacillus* strains (Vahabnezhad et al. 2013). Analysis of the results of 74 different controlled clinical trials showed that the use of probiotic and symbiotic in children between 0 and 18 years old was not linked to an increased health risk (Van den Nieuwboer et al. 2015). *L. rhamnosus* and *L. casei*, which are commonly present in the commercial probiotic products, belong to the strains most frequently isolated during bacteremia caused by *Lactobacillus* bacteria (Salminen et al. 2004). These *Lactobacillus* species may have a greater potential to translocate and therefore, they could be more pathogenic than other species (Liong 2008). Other probiotic microorganisms directly related to bacteremia and endocarditis are: *L. plantarum*, *L. paracasei*, *L. salivarius*, *L. acidophilus* and many other lactobacilli. Moreover, *Lactococcus lactis*, *Leuconostoc*, *Pediococcus* and *Bifidobacterium* have also been shown to induce bacteremia and endocarditis (Snydman 2008). The studies by Harty and coworkers (Harty et al. 1993; Harty et al. 1994) showed that *L. rhamnosus* strains, isolated from patients with endocarditis, possess capability to aggregate platelets and to adhere to fibronectin, fibrinogen and collagen. This aggregation could be related to proteins of the intestinal epithelium. In addition, *L. rhamnosus* and *L. paracasei* subsp. *paracasei* produce enzymes that degrade human glycoproteins and fibrin clots, and this observation suggests that these molecules may participate in the development of infective endocarditis (Liong et al. 2008). Kochan et al. (2011) described a case of sepsis caused by *L. rhamnosus* in a woman with a heart valve. A bacterial translocation probably occurred by a leaky intestinal barrier and led to heart failure in this patient (Kochan et al. 2011). Some cases of *Lactobacillus*-related bacteremia have been reported, including *L. rhamnosus* GG, *L. casei* and *L. acidophilus* (De Groote et al. 2005; Ledoux et al. 2006; Vahabnezhad et al. 2013). Moreover, nine cases of sepsis have been described, associated with *S. boulardii, L. rhamnosus* GG, *Bifidobacterium breve*, *Bacillus subtilis* or combination of probiotic bacteria (Doron and Snydman 2015). Fungemia, caused by *Saccharomyces cerevisiae* var. *boulardii* is the most commonly reported single infection (33 reports) (Doron and Snydman 2015). Among 89 strains from blood samples, analyzed by pulse-field gel electrophoresis, eleven strains had identical PFGE patterns as the probiotic strain *L. rhamnosus* GG (Salminen et al. 2004). However, the other studies showed that pathogenic *L. rhamnosus* GG-like strains isolated from the blood cultures, were phenotypically different from probiotic *L. rhamnosus* GG (Ouwehand et al. 2004). Cases of deaths of healthy people caused by the intake of probiotic bacteria are very rare. The percentage of lethal infections caused by *Lactobacillus* is very low; however, these bacteria may infrequently cause bacteremia or endocarditis (Doron and Snydman 2015).

The probiotic strains have different antimicrobial susceptibility. The high doses of penicillin or ampicillin with or without aminoglycosides are most often used in treatment of lactobacilli infections. The retrospective study of 45 cases of bacteremia, demonstrated that 100% of lactobacilli were susceptible to ampicillin, clindamycin and erythromycin, 96% were susceptible to penicillin and 67% to gentamycin (Sherid et al. 2016). On the other hand, bifidobacteria are generally susceptible to β-lactams, glycopeptides and erythromycin (Moubareck et al. 2005), while fungemias caused by *Saccharomyces* strain may be treated with fluconazole, amphotericin B or voriconazole (Burkhardt et al. 2005).

Stimulation of the immune system

Probiotic strains may modulate the immune response of individuals, and this may result in the increased response to vaccines or allergens. Besides, these strains also have an effect on both cellular and humoral responses, and affect the secretion of cytokines (Senok et al. 2005). Side effects, such as fever or arthritis, can be caused by the presence of peptide-glycan-polysaccharides, which are components of the bacterial cell wall, e.g. of the *Lactobacillus* genus (Marteau 2001). The immune system of healthy and immunocompromised individuals may react differently to probiotic bacteria. For example, probiotic bacteria may exert a stimulating effect on phagocytosis in healthy people, whereas for people with allergies, this effect can be opposite (Senok et al. 2005). The immunomodulatory effect can also depend on the dose of probiotic used (Tsai et al. 2012). Despite the lack of direct reports on the probiotics harmful for immunocompromised individuals, it seems important to continue research on the efficacy and safety of probiotics (Sanders et al. 2010; Doron and Snydman 2015).

Detrimental metabolic effect

Probiotic bacteria during colonization of the small bowel deconjugate and dehydroxylate bile salts what could results in diarrhea and intestinal lesions (Agostoni and Salvini 2009). Since probiotic strains can produce bile salt hydrolase (BSH), the deconjugated bile salts could be accumulated, and then altered into harmful secondary bile acids by intestinal microbiota.

The accumulation of these cytotoxic compounds in the enterohepatic circulation could increase the risk of cholestasis and colorectal cancer (Tan et al. 2007). The harmful effects exerted by BSH on humans have not yet been accurately described (Ooi and Liong 2010). The probiotic *L. acidophilus* and *Bifidobacterium* spp., the strains derived from fermented milk products, may also convert the primary bile salts to secondary bile salts. If decarboxylation and dehydroxylation processes occur in excess, then there may be a potential risk to the patient's health (Marteau et al. 1995).

Other deleterious metabolic effect is D-lactate production by probiotic strains (Doron and Snydman 2015). Humans produce the L(+)-isomer of lactic acid, while the presence of D(–)-lactate is the result of bacterial metabolism or transformation of L(+)-lactate by a bacterial DL-lactate racemase (Hove and Mortensen 1995; Sanders et al. 2010). Some of the *Lactobacillus* strains may produce L(+)-lactic acid as well as D(–)-lactic acid and transform one isomer into the other (Vitetta et al. 2017). In healthy humans, the increased level of D-lactic acidosis is rarely observed (Vitetta et al. 2017), but in children with short bowel syndrome the blood concentration of D-lactic acid is high during an exacerbation of symptoms (Bongaerts 2000; Sanders et al. 2010). Five reports of D-lactic acidosis can be found in the literature (Doron and Snydman 2015), of which two concern the patients with short bowel syndrome, both proceeded by the administration of probiotics (Łukasik et al. 2018). Infants may be more vulnerable to D-lactic acidosis, because of weaken barrier function of the intestinal tract and reduced ability of renal excretion (Sanders et al. 2010; Łukasik et al. 2018). In patients at risk of developing D-lactic acidosis, especially those with former bowel surgery and subsequent gut syndrome, and in newborns and neonates, administration of probiotics which may produce D(–)-lactate should be handled very carefully (Sanders et al. 2010).

Probiotic deleterious metabolic activities, such as degradation of mucin may also contribute to potential side effects after their consumption. In this case, the number of microorganisms that translocate through the small bowel is increased, possibly causing gastrointestinal disturbances including intestinal inflammation. One hypothesis states that the accumulation of probiotics in the gastrointestinal tract could result in increased risk of intestinal mucus degradation, but it was not confirmed. Ruseler-van-Embeden et al. (1995) conducted experiments on germ-free rats. They studied the degradation of mucus glycoprotein by *Lactobacillus casei* strain GG, *L. acidophilus*, *B. bifidum* and lactic culture isolated from fermented products, but did not observe any degradation of intestinal mucus glycoproteins or damage of the intestinal mucus layer (Ruseler-van Embden et al. 1995). Moreover, Abe et al. (2010) did not observe translocation, damage of epithelial cells or any changes of the mucosal layer in the ileum, cecum and colon.

Potential transfer of genes

Another aspect concerning the safety of bacteria used as probiotics is the potential transfer of the antibiotic resistance genes between probiotics and other commensal or pathogenic bacteria that occur in the gastrointestinal tract (Teuber et al. 1999; Salyers et al. 2004; Snydman 2008; Nawaz et al. 2011). It has been reported that over 68% of probiotic strains were resistant to two or more antibiotics. Moreover, *Bacillus* strains from some probiotic products display even a high-level resistance. Lactic acid bacteria are naturally resistant to some antibiotics (Gueimonde et al. 2004). Many lactobacilli strains, apart from *L. delbrueckii* subsp. *bulgaricus*, *L. acidophilus*, *L. johnsonii* and *L. crispatus* are naturally resistant to vancomycin (Charteris et al. 1998; Balletti et al. 2009; Liu et al. 2009; Shao et al. 2015; Guo et al. 2017); however, the genes responsible for this resistance are chromosomally located and not easily transferable to other genera (Tynkkynen et al. 1998; Shao et al. 2015). *Lactobacillus* species are often resistant to aminoglycosides, monobactams and fluoroquinolones (Zheng et al., 2017). Guo et al. (2017) reported that 70% of the *Lactobacillus* strains tested were resistant to ciprofloxacin. The resistance of LAB strains to tetracycline, erythromycin, chloramphenicol, streptomycin, lincosamides and streptogramins may be due to the presence of plasmids bearing the genes encoding resistance to these antibacterial agents (Doron and Snydman 2015). The most commonly found resistance genes in *Lactobacillus* species are *tet*(M) and *erm*(B), responsible for tetracycline and erythromycin resistance, respectively. Moreover, the *cat* gene encoding chloramphenicol acetyltransferase was also present in LAB (Fukao and Yajma 2012). The *tet*(M) genes localized in *Lactobacillus* strains of various species, derived from fermented food possess a high similarity to the genes present in the pathogenic meningococcal or *Staphylococcus aureus* strains (Gevers et al. 2003a; Gevers et al. 2003b). The resistance plasmids carrying the *tet*(M) and *erm*(B) genes have been found in *L. reuteri*, *L. fermentum*, *L. acidophilus* and *L. plantarum* isolated from raw meat, silage and feces of animals (Snydman 2008). The isolates of *Leuconostoc* and *Pediococcus* can accept broad host range antibiotic resistance plasmids from *Lactococcus* species (Dessart and Steenson 1991). Moreover, *in vitro* conjugation transfer may occur from *Enterococcus* to *Lactococcus* and *Lactobacillus* (Doron and Snydman 2015). In some *Lactobacillus* strains (e.g., *L. plantarum*, *L. reuteri*, *L. fer-*

mentum) a transmission of antibiotic resistance genes to other LAB strains occur via the pAMβ plasmid (Schjørring and Krogfelt 2011). Analysis of gene transfer from 44 strains of *L. acidophilus*, 14 strains of *L. delbrueckii,* six strains of *L. casei rhamnosus*, five strains of *L. plantarum*, one strain of *L. helveticus*, one strain of *L. brevis* and one strain of *L. fermentum*, showed that only a single strain of *L. helveticus* and one *L. brevis* accepted the plasmid with low efficiency (Morelli et al. 1988). Sybesma et al. (2013) compared the genes from three bacterial isolates derived from dairy products containing the *L. rhamnosus* GG strains with a reference strain from the ATCC collection (*L. rhamnosus* 53103). In two of three strains tested, the lack of DNA fragments encoding 34 and 84 genes that affect the adhesion of these strains and their persistence in the gastrointestinal tract was observed (Sybesma et al. 2013). These examples underline the potential for genetic variation in LAB and other probiotic bacteria, and suggest that these changes may occur in the microbial cultures used for commercial processes (Sanders et al. 2014).

The risk of transfer of antibiotic resistance genes may be an argument against use of probiotic, for example in aquaculture. On the other hand, some antibiotic resistance phenotypes, for example resistance of few lactobacilli strains to vancomycin (Tynkkynen et al. 1998), or resistance represented by *Streptomyces* strains, are not easily transferable to other genera (Tan et al. 2016). Bacterial strains used as probiotic drugs, foods or dairy products should be systematically controlled in order to detect the antibiotic resistance genes that could be transferable to pathogenic bacteria. According to the guidelines of European Food Safety Authority (EFSA) all bacterial strains, used as feed additives, should be tested to determine their sensitivity to commonly used antibiotics (European Food Safety Authority 2012).

Identification

The correct identification of strains is extremely important for safety, growth conditions and metabolic properties of a given strain (FAO/WHO 2002). The correct classification of strains in a microbiome is extremely important at the time of preparation of the probiotic since this information is crucial for assessment of the stability of the strain, and to perform comparison with clinical isolates in the case of infection (Sanders et al. 2010). Even if the strain of probiotic bacteria is considered as safe, it still can cause bacteremia (Van den Nieuwboer et al. 2014) as opportunistic bacteria.

Microbiological examination of probiotic products showed that they might contain some microorganisms that were not indicated on the label (Hoa et al. 2000; Temmerman et al. 2002; Coeuret et al. 2004; Millazzo et al. 2004; Masco et al. 2005; Zawistowska-Rojek et al. 2016). The presence of these microorganisms indicates an inadequate control of production or insufficient control procedures. Some of the available probiotic products are labeled poorly, with lack of information not only on the classification of the strains included, but also about the excipients present in product, such as cow's milk protein, which may be an allergen for some people (Moneret-Vutrin et al. 2006; Lee et al. 2007).

Conclusion

The major risk factor in safe applications of probiotic microorganisms is the lack of knowledge on their activity. The interactions between intestinal microbes and the host have a major influence on the overall health condition. The suitable characteristics of relationships between probiotic structure and function would reduce the possibility of side effects. Generally, probiotic bacteria have a beneficial effect on the digestive system but in some cases they may facilitate the translocation or induce infections themselves. Due to the fact that the adverse effects caused by probiotics are documented, it is necessary to fully understand the mechanisms of activity of probiotic bacteria. In addition, the differences in activity of a single strain or a mixture of strains of different species or even genera should also be taken into account before the probiotic use in humans (Sanders et al. 2010).

Literature

Abe F, Muto M, Yaeshima T, Iwatsuki K, Aihara H, Ohashi Y, Fujisawa T. 2010. Safety evaluation of probiotic bifidobacteria by analysis of mucin degradation activity and translocation ability. Anaerobe. 16:131–136.

Agostoni C, Salvini F. 2009. Probiotics in Infant Dietetics. In: Michail S, Sherman PM, editors. Probiotics in Pediatric Medicine. New York (USA): Humana Press.

Ambesh P, Stroud S, Franzova E, Gotesman J, Sharma K, Wolf L, Kamholz S. 2017. Recurrent *Lactobacillus* bacteremia in a patient with leukemia. J Investig Med High Impact Case Rep. 5:1–3.

Ammor MS, Florez AB, Mayo B. 2007. Antibiotic resistance in non-enterococcal lactic acid bacteria and bifidobacteria. Food Microbiol. 24:559–570.

Ashraf A, Shah NP. 2011. Antibiotic resistance of probiotic organisms and safety of probiotic dairy products. Int Food Res J. 18:837–853.

Bayer AS, Chow AW, Betts D, Guze LB. 1978. Lactobacillemia: report of 9 cases: important clinical and therapeutic considerations. Am J Med. 64:808–813.

Belletti N, Gatti M, Bottari B, Neviani E, Tabanelli G, Gardinni F. 2009. Antibiotic resistance of lactobacilli isolated from two Italian hard cheeses. J Food Prot. 72:2162–2169.

Berg RD. 1985. Bacterial translocation from the intesties. Exp Anim. 34:1–16.

Berg RD. 1992. Translocation and the indigenous gut flora. In: Fuller R. Probiotics: The Scientific Basis. London (UK): Chapman & Hall. p. 55–85.

Bongaerts G, Bakkeren J, Severijnen R, Sperl W, Willems H, Naber T, Wevers R, van Meurs A, Tolboom J. 2000. Lactobacilli and acidosis in children with short small bowel. J Pediatr Gastroenterol Nutr. 30:288–293.

Borriello SP, Hammes WP, Holzapfel W, Marteau P, Schhrezenmeir J, Vaara M, Valtonen V. 2003. Safety of probiotics that contain lactobacilli and bifidobacteria. Clin Infect Dis. 36:775–780.

Boyle RJ, Robins-Browne RM, Tang ML. 2006. Probiotic use in clinical practice: what are the risks? Am J Clin Nutr. 83:1256–1264.

Brook I. 1996. Isolation of non-sporing anaerobic rods from infections in children. J Med Microbiol. 45:21–26.

Brughton RA, Gruber WC, Haffar AA, Baker CJ. 1983. Neonatal meningitis due to *Lactobacillus*. Pediatr Infect Dis J. 2:382–384.

Brumfitt W, Ludlam H, Hamilton-Miller JMT, Gooding A. 1981. Lactobacilli do not cause frequency and dysuria a syndrome. Lancet. 2:393–395

Burkhardt O, Köhnlein T, Pletz M, Welte T. 2005. *Saccharomyces boulardii* induced sepsis: successful therapy with voriconazole after treatment failure with fluconazole. Scand J Inf Dis. 37:69–72.

Charteris WP, Kelly PM, Morelli L, Collins JK. 1998. Antibiotic susceptibility of potentially probiotic *Lactobacillus* species. J Food Prot. 61:1636–1643.

Coeuret V, Gueguen M, Vernoux JP. 2004. Numbers and strains of lactobacilli in some probiotic products. Int J Food Microbiol. 97:147–156.

De Groote MA, Frank DN, Dowell E, Glode MP, Pace NR. 2005. *Lactobacillus rhamnosus* GG bacteremia associated with probiotic use in a child with short gut syndrome. Ped Infect Dis J. 24:278–280.

Dessart SR, Steenson LR. 1991. High frequency intergeneric and intrageneric conjugal transfer of drug resistance plasmids in *Leuconostoc mesenteroides* ssp *cremoris.* J Dairy Sci. 74:2912–2919.

Dickgieber U, Weiss N, Fritsche D. 1984. *Lactobacillus gasseri* as the cause of septic urinary infection. Infection. 12:14–16.

Doron S, Snydman DR. 2015. Risk and safety of probiotics. Clin Infect Dis. 60(Suppl 2):129–134.

European Food Safety Authority. 2012. Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance. EFSA J. 10(2740):1–10.

Fukao M, Yajima N. 2012. Assessment of antibiotic resistance in probiotic lactobacilli. In: Pana M, editor. Antibiotic resistant bacteria – A continuous challenge in the new millennium. London (UK): IntechOpen [Internet]. doi: 10.5772/30903; [cited 2017 December 20]. Available from: http://www.intechopen.com/books/antibiotic-resistant-bacteria-a-continuous-challenge-in-the-new-millennium/assessment-of-antibiotic-resistance-in-probiotic-strains

Gevers D, Danielsen M, Huys G, Swings J. 2003a. Molecular characterization of *tet*(M) genes in *Lactobacillus* isolates from different types of fermented dry sausage. Appl Environ Microbiol. 69:1270–1275.

Gevers D, Huys G, Swings J. 2003b. *In vitro* conjugal transfer of tetracycline resistance from *Lactobacillus* isolates to other Gram-positive bacteria. FEMS Microbiol Lett. 225:125–130.

Global Market Insights [Internet]. 2017. Los Ageles (USA): Globe-Newswire; [cited 2018 March 14]. Available from: globenewswire.com/news-release/2017/10/10/1143574/0/en/Probiotics-Market-to-exceed-65bn-by-2024-Global-Market-Insights-Inc.html

Gueimonde M, Ouwehand AC, Salminen S. 2004. Safety of probiotics. Scand J Nutr. 48:42–48.

Guo H, Pan L, Li L, Lu J, Kwok L, Menghe B, Zhang H, Zhang W. 2017. Characterization of antibiotic resistance genes from lactobacillus isolated from traditional dairy products. J Food Sci. 82:724–730.

Harty DWS, Oakey HJ, Patrikakis M, Hume EBH, Knox KW. 1994. Pathogenic potential of lactobacilli. Int J Food Microbiol. 24:179–189.

Harty DWS, Patrikakis M, Knox KW. 1993. Identification of *Lactobacillus* strains from patients with infective endocarditis and comparison of their surface-associated properties with those of other strains of the same species. Microb Ecol Health Dis. 6:191–201.

Hempel S, Newberry S, Ruelaz A, Wang Z, Miles JN, Suttorp MJ, Johansen B, Shanman R, Slusser W, Fu N, et al. 2011. Safety of probiotics used to reduce risk and prevent or treat disease. Evid Rep Technol Assess. 200:1–645.

Hill C, Guarner F, Reid G, Gibson GR, Merenstein DJ, Pot B, Morelli L, Canani RB, Flint HJ, Salminen S. 2014. Expert consensus document "The international scientific association for probiotics and prebiotics consensus statement on the scope and appropriate use of the term probiotic". Nat Rev Gastroenterol Hepatol. 11:506–514.

Hoa NT, Baccigalupi L, Huxham A, Smertenko A, Van PH, Ammendola S, Ricca E, Cutting AS. 2000. Characterization of *Bacillus* species used for oral bacteriotherapy and bacterioprophylaxis of gastrointestinal disorders. Appl Environ Microbiol. 66: 5241–5247.

Horwitch CA, Furseth HA, Larson AM, Jones TL, Olliffe JF, Spach DH. 1995. Lactobacillemia in three patients with AIDS. Clin Infect Dis. 21:1460–1462.

Hove H, Mortensen PB. 1995. Influence of intestinal inflammation (IBD) and small and large-bowel length on fecal short-chain fatty-acids and lactate. Dig Dis Sci. 40:1372–1380.

Ishibashi N, Yamazaki S. 2001. Probiotics and safety. Am J Clin Nutr. 73(Suppl):465S–470S.

Johnson BR, Klaenhammer TR. 2014. Impact of genomics on the field of probiotic research: historical perspectives to modern paradigms. Antoine van Leeuwenhoek. 106:141–156.

Kalima P, Masterton RG, Roddie PH, Thomas AE. 1996. *Lactobacillus rhamnosus* infection in a child following bone marrow transplant. J Infect. 32:165–167.

Kochan P, Chmielarczyk A, Szymaniak L, Brykczynski M, Galant K, Zych A, Pakosz K, Giedrys-Kalemba S, Lenouvel E, Heczko PB. 2011. *Lactobacillus rhamnosus* administration causes sepsis in a cardiosurgical patient – is the time right to revise probiotic safety guidelines? Clin Microbiol Infect. 17:1589–1592.

Kunz AN, Noel JM, Fairchok MP. 2004. Two cases of *Lactobacillus* bacteremia during probiotc treatment of short gut syndrome. J Ped Gastroent Nutr. 38:457–458.

Land MH, Rouster-Stevens K, Woods CR, Cannon ML, Cnota J, Shetty AK. 2005. *Lactobacillus* sepsis associated with probiotic therapy. Pediatrics. 115:178–181.

Ledoux D, Labombardi VJ, Karter D. 2006. *Lactobacillus acidophilus* bacteremia after use of a probiotic in a patient with AIDS and Hodgkin`s disease. Int J STD AIDS. 17:280–282.

Lee TT, Morisset M, Astier C, Moneret-Vautrin DA, Cordebar V, Beaudouin E, Codreanu F, Bihain BE, Kanny G. 2007. Contamination of probiotic preparations with milk allergens can cause anaphylaxis in children with cow's milk allergy. J Allergy Clin Immunol. 119:746–747.

Lilly DM, Stillwell RH. 1965. Growth promoting factors produced by probiotics. Science. 147:747–748.

Liong MT. 2008. Safety of probiotics: translocation and infection. Nutr Rev. 66:192–202.

Liu Ch, Zhang Z, Dong K, Yuan J, Guo X. 2009. Antibiotic resistance of probiotic strains of lactic acid bacteria isolated from marketed foods and drugs. Biomed Env Sci. 22:401–412.

Łukasik J, Salminen S, Szajewska H. 2018. Rapid review shows that probiotics and fermented infant formulas do not cause D-lactic acidosis in healthy children. Acta Paediatr. 107:1322–1326.

Masco L, Huys G, De Brandt E, Temmerman R, Swings J. 2005. Culture-dependent and culture-independent qualitative analysis of probiotic products claimed to contain bifidobacteria. Int J Food Microbiol. 102:221–230.

Mathur S, Singh R. 2005. Antibiotic resistance in food lactic acid bacteria – a review. Int J Food Microbiol. 105:281–295.

McFarland LV. 2015. From yaks to yogurt: the history, development, and current use of probiotics. Clin Infect Dis. 60(S2):S85–S90.

Metchnikoff E. 1908. The prolongation of life. Optimistic studies. New York (USA): Putnam's Sons. p. 161–183.

Milazzo I, Speciale A, Musumeci R, Fazio D, Blandino G. 2006. Identification and antibiotic susceptibility of bacterial isolates from probiotic products available in Italy. New Microbiol. 29: 281–291.

Moneret-Vautrin DA, Morisset M, Cordebar V, Codreanu F, Kanny G. 2006. Probiotics may be unsafe in infants allergic to cow's milk. Allergy. 61:507–508.

Morelli L, Sarra PG, Bottazzi V. 1988. *In vivo* transfer of pAM beta 1 from *Lactobacillus reuteri* to *Enterococcus faecalis*. J Appl Bacteriol. 65:371–375.

Moubareck C, Gavini F, Vaugien L, ButelMJ, Doucet-Populaire F. 2005. Antimicrobial susceptibility of Bifidobacteria. J Antimicrob Chemother. 55:38–44.

Nawaz M, Wang J, Zhou A, Ma C, Wu X, Moore JE, Millar BC, Xu J. 2011. Characterization and transfer of antibiotic resistance in lactic acid bacteria from fermented food products. Curr Microbiol. 62:1081–1089.

Ooi LG, Liong MT. 2010. Cholesterol-lowering effects of probiotics and prebiotics: a review of *in vivo* and *in vitro* findings. Int J Mol Sci. 11:2499–2522.

Ouwehand AC, Saxelin M, Salminen S. 2004. Phenotypic differences between commercial *Lactobacillus rhamnosus* GG and *L. rhamnosus* strains recovered from blood. Clin Infect Dis. 39: 1858–1860.

Patel R, Cockerill FR, Porayko MK, Osmon DR, Ilstrump DM, Keating MR. 1994. Lactobacillemia in liver transplant patients. Clin Infect. 18:207–212.

Report FAO/WHO. 2001. Report of a Joint FAO/WHO Expert Consultation on Evaluation of Health and Nutritional Properties of Probiotics in Food including Powder Milk with Live Lactic Acid Bacteria [Internet]. p. 1–29; [cited 2017 November 15]. Available from: http://www.fao.org/tempref/docrep/fao/meeting/009/y6398e.pdf

Report FAO/WHO. 2002. Report of a Joint FAO/WHO Working Group Report on Drafting Guidelines for the Evaluation of Probiotics in Food [Internet]. p. 36–48; [cited 2017 November 15]. Available from: http://www.who.int/foodsafety/fs_management/en/probiotic_guidelines.pdf

Ruseler-van Embden JGH, van Lieshout LMC, Gosselink MJ, Marteau P. 1995. Inability of *Lactobacillus casei* strain GG, *L. acidophilus*, and *Bifidobacterium bifidum* to degrade intestinal mucus glycoproteins: clearing the way for mucosa-protective therapy. Scand J Gastroenterol. 30:675–680.

Saarela M, Mogensen G, Fonden R, Matto J, Mattila-Sandholm T. 2000. Probiotic bacteria: safety, functional and technological properties. J Biotechnol. 84:197–215.

Salminen MK, Rautelin H, Tynkkynen S, Poussa T, Saxelin M, Valtonen V, Järvinen A. 2004. *Lactobacillus* bacteremia, clinical signifiance, and patient outcome, with special focus on probiotic *L. rhamnosus* GG. Clin Infect Dis. 38:62–69.

Salminen MK, Tynkkynen S, Rautelin H, Saxelin M, Vaara M, Ruutu P, Sarna S, Valtonen V, Järvinen A. 2002. *Lactobacillus* bacteremia during a rapid increase in probiotic use f *Lactobacillus rhamnosus* GG in Finland. Clin Infect Dis. 35:1155–1160.

Salyers AA, Gupta A, Wang Y. 2004. Human intestinal bacteria as reservoirs for antibiotic resistance genes. Trends Microbiol. 12:412–416.

Sanders ME, Akkermans LMA, Haller D, Hammerman C, Heimbach J, Hormannsperger G, Huys G, Levy DD, Lutgendorff F, Mack D, et al. 2010. Safety assessment of probiotic for human use. Gut Microbes. 1:164–185.

Sanders ME, Klaenhammer TR, Ouwehand AC, Pot B, Johanses E, Heimbach JT, Marco ML, Tenilä J, Ross RP, Franz C, et al. 2014. Effects of genetic, processing, or product formulation changes on efficacy and safety of probiotics. Ann N.Y. Acad Sci. 1309:1–18.

Schjørring S, Krogfelt KA. 2011. Assessment of bacterial antibiotic resistance transfer in the gut. Int J Microbiol [Internet]:1–10; [cited 2017 December 17]. Available from: http://dx.doi.org/10.1155/2011/312956

Schlegel L, Lemerle S, Geslin P. 1998. *Lactobacillus* species as opportunistic pathogens in immunocompromised patients. Eur J Clin Microbiol Infect. 17:887–888.

Senok AC, Ismaeel AY, Botta GA. 2005. Probiotics: facts and myths. Clin Microbiol Infect. 11:958–966.

Shao Y, Zhang W, Guo H, Pan L, Zhang H, Sun T. 2015. Comparative studies on antibiotic resistance in *Lactobacillus casei* and *Lactobacillus plantarum*. Food Control. 50:250–258.

Sharpe ME, Hill LR, Lapage SP. 1973. Pathogenic lactobacilli. J Med Microbiol. 6:281–286.

Sherid M, Samo S, Sulaiman S, Husein H, Sifuentes H, Sridhar S. 2016. Liver abscess and bacteremia caused by lactobacillus: role of probiotics? Case report and review of the literature. BMC Gastroenterol. 16:138.

Sherman ME, Albrecht M, DeGirolami PC, Kirkley SA, Wolf B, Eliopoulos GM, Rohrer RJ, Monaco AP. 1987. An unusual case of splenic abscess and sepsis in an immunocompromised host. Am J Clin Pathol. 88:659–662.

Snydman DR. 2008. The safety of probiotics. Clin Infect Dis. 46:S 104–111.

Sriskandan S, Lacey S, Fisher L. 1993. Isolation of vancomycin-resistant lactobacilli from three neutropenic patients with pneumonia. Eur J Clin Microbiol Infect Dis. 12:649–650.

Sybesma W, Molenar D, van Ijcken W, Venema K, Kort R. 2013. Genome instability in *Lactobacillus rhamnosus* GG. Appl Environ Microbiol. 79:2233–2239.

Tan KP, Yang M, Ito S. 2007. Activation of nuclear factor (erythroid-2 like) factor 2 by toxic bile acids provokes adaptive defense responses to enhance cell survival at the emergence of oxidative stress. Mol Pharmacol. 72:1380–1390.

Tan LT-H, Chan K-G, Lee L-H, Goh B-H. 2016. *Streptomyces* bacteria as potential probiotics in aquaculture. Front Microbiol. 7:79.

Temmerman R, Pot B, Huys G, Swings J. 2002. Identification and antibiotic susceptibility of bacterial isolates from probiotic products. Int J Food Microbiol. 81:1–10.

Teuber M, Meile L, Schwarz F. 1999. Acquired antibiotic resistance in lactic acid bacteria from food. Antoine Van Leeuwenhoek. 76:115–137.

Thompson C, McCarter YS, Krause PJ, Herson VC. 2001. *Lactobacillus acidophilus* sepsis in a neonate. J Perinatol. 21:258–260.

Tissier H. 1906. Treatment of intestinal infections using bacterial flora of the intestine. Crit Rev Soc Biol. 60:359–361.

Tsai Y-T, Cheng P-C, Pan T-M. 2012. The immunomodulatory effects of lactic acid bacteria for improving immune functions and benefits. Appl Microbiol Biotechnol. 96:853–862.

Tynkkynen S, Singh KV, Varmanen P. 1998. Vancomycin resistance factor of *Lactobacillus rhamnosus* GG in relation to enterococcal vancomycin resistance (*van*) genes. Int J Food Microbiol. 41:195–204.

Vahabnezhad E, Mochon AB, Wozniak LJ, Ziring DA. 2013. *Lactobacillus* bacteremia associated with pobiotic use in a pediatric patient with ulcerative colitis. J Clin Gastroenterol. 47: 437–439.

Van den Nieuwboer M, Brummer RJ, Guarner F, Cabana M, Morelli L, Claassen E. 2015. Safety of probiotics and synbiotics in children under 18 years of age. Benef Microbes. 6:615–630.

Van den Nieuwboer M, Claassen E, Morelli L, Guarner F, Brummer RJ. 2014. Probiotic and synbiotic safety in infants under two years of age. Benef Microbes. 5:45–60.

Van Leeuwen PAM, Boermeester MA, Houdijk APJ, Ferwerda ChC, Cuesta MA, Meyer S. 1994. Clinical significance of translocation. Gut. 35(Suppl):S28–S34.

Vankerckhoven V, Huys G, Vancanneyt M, Vael C, Klare I, Romond MB, Enteza JM, Moreillon P, Wind RD, Knol J, et al. 2008. Biosafety assessment of probiotics used for human consumption: recommendations from the EU-PROSAFE project. Trend Food Sci Tech. 19:102–114.

Venugopalan V, Shriner KA, Wong-Beringer A. 2010. Regulatory oversight and safety of probiotic use. Emerging Infect Dis. 11:1661–1665.

Vitetta L, Coulson S, Thomsen M, Nguyen T, Hall S. 2017. Probiotics, D-lactic acidosis, oxidative stress and strain specificity. Gut Microb. 8:311–322.

Zawistowska-Rojek A, Zaręba T, Mrówka A, Tyski S. 2016. Assessment of the microbiological status of probiotic products. Pol J Microb. 65:97–104.

Zheng M, Zhang R, Tian X, Zhou X, Pan X, Wong A. 2017. Assessing the risk of probiotic dietary supplements in the context of antibiotic resistance. Front Microbiol. 8:908.

Polish Journal of Microbiology
2018, Vol. 67, No 3, 259–272
https://doi.org/10.21307/pjm-2018-048

MINIREVIEW

Secondary Metabolites of Actinomycetes and their Antibacterial, Antifungal and Antiviral Properties

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Submitted 25 May 2018, revised 1 July 2018, accepted 18 July 2018

Abstract

The growing resistance of microorganisms towards antibiotics has become a serious global problem. Therapeutics with novel chemical scaffolds and/or mechanisms of action are urgently needed to combat infections caused by multidrug resistant pathogens, including bacteria, fungi and viruses. Development of novel antimicrobial agents is still highly dependent on the discovery of new natural products. At present, most antimicrobial drugs used in medicine are of natural origin. Among the natural producers of bioactive substances, Actinobacteria continue to be an important source of novel secondary metabolites for drug application. In this review, the authors report on the bioactive antimicrobial secondary metabolites of Actinobacteria that were described between 2011 and April 2018. Special attention is paid to the chemical scaffolds, biological activities and origin of these novel antibacterial, antifungal and antiviral compounds. Arenimycin C, chromopeptide lactone RSP 01, kocurin, macrolactins A1 and B1, chaxamycin D as well as anthracimycin are regarded as the most effective compounds with antibacterial activity. In turn, the highest potency among selected antifungal compounds is exhibited by enduspeptide B, neomaclafungins A-I and kribelloside D, while ahmpatinin ^{i}Bu, antimycin A1a, and pentapeptide 4862F are recognized as the strongest antiviral agents.

Key words: bioactive, secondary metabolites, actinomycetes, antibacterial activity, *Streptomyces* sp.

Introduction

Natural products play a predominant role in the development of new therapeutic agents (Newman and Cragg 2016). Actinobacteria represent the most prominent group of microorganisms, which produce bioactive compounds. They synthesize approximately two-thirds of all naturally derived antibiotics currently used in medicine, veterinary practice and agriculture. Majority of these molecules originate from *Streptomyces* genus (Barka et al. 2016; Chater 2016).

A traditional approach in obtaining novel bioactive agents, especially with unique chemical structures and biological significance, relies on distinct microorganisms isolated from different, often secluded, environments. Actinobacterial strains commonly derive from soil (Guo et al. 2015), but they are also abundantly present in seas and oceans (Hassan et al. 2015; Xu et al. 2017). Moreover, extreme habitats such as caves (Jiang et al. 2015), deserts (Goodfellow et al. 2017) or Antarctic ecosystems (Lee et al. 2012) are recognized as valuable sources of actinomycetes producing novel metabolites of pharmacological importance (Solecka et al. 2013; Singh et al. 2018). Biosynthesis of secondary metabolites depends on the growth conditions of each strain. For years researchers have been applying different modifications in nutrients and physicochemical factors during fermentation processes to optimize the production of bioactive compounds (Rajnisz et al. 2016). Currently, modeling and analysis of fermentation processes is performed by the statistical optimization approach, e.g. response surface methodology, which enables enhanced production of antibiotics, enzymes and probiotics (Latha et al. 2017).

Genome sequencing can also be applied to detect the genes responsible for production of secondary metabolites besides those that were isolated under standard cultivation conditions (Thong et al. 2015). Most

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microbially derived natural products are produced via metabolic pathways encoded by adjacent chromosomal genes: biosynthetic gene clusters (BGCs). BGCs encode enzymes, regulatory proteins and transporters that are necessary to produce, process and export a metabolite (Medema and Fischbach 2015). On average, BGCs encompass 1.64 Mbp (16% of actinomycete coding capacity), encoding 35 secondary metabolites. The largest number of natural products was determined to be encoded by *Kutzneria albida*, *S. bingchenggensis* and *S. rapamycinicus* strains, which devote 2.5–3.09 Mbp (> 20% of coding capacity) to encode 48–53 secondary metabolites (Wink et al. 2017). Most of BGCs are cryptic or poorly expressed under laboratory conditions (Baltz 2011). However, many methods have been developed to activate the actinobacterial secondary metabolism, including combined cultures and use of goadsporins (Onaka 2017). An effective strategy in the development of strains with enhanced secondary metabolism was established with the recent advancement of whole-genome sequencing, systems biology and genetic engineering. In this aspect, the "-omics" technologies (genomics, transcriptomics, proteomics and metabolomics) together with bioinformatic are especially useful tools in inducing the overproduction of actinomycetes secondary metabolites. The past few years were an exciting time for metabolic engineering of actinomycetes as several novel in silico methods to automate the analysis of secondary metabolism in bacterial genomes (e.g. antibiotics & Secondary Metabolite Analysis Shell, antiSMASH) had been introduced (Medema et al. 2011).

In this review, we present secondary metabolites of Actinobacteria, which possess antibacterial, antifungal and antiviral activities. Their origin and chemical features are also reported. We draw special attention to novel agents described from 2011 to April 2018, displaying minimum inhibitory concentrations (MICs) less than or equal to 10 μg/ml. Additionally, we discuss on the previously described compounds that were recently characterized with new properties and function. The present article is a thematic continuation of a review published in 2012 (Solecka et al. 2012).

Antibacterial activity

Nowadays, antibiotic resistance of microorganisms is one of the biggest threats to global health, food security and development. The World Health Organization (WHO) Global Report on the surveillance of antimicrobial resistance has established that bacterial resistance to commonly used drugs in infection treatment has reached alarming levels in many parts of the world (WHO 2014). In 2017, WHO released its first list of most concerning "priority pathogens" for human health – a catalogue of twelve families of bacteria for which new antibiotics are urgently needed (WHO 2017). According to O'Neill's independent review (2016) about 700 000 people around the world die annually due to drug-resistant infections. If current trends continue, such infections could entail the death of 10 million people a year by 2050 (O'Neill 2016). In this context, the discovery of new bioactive compounds, particularly those with new modes of action, is not only needed for modern medicine but absolutely required to avoid future pandemics.

Below authors present the major chemical structural groups of newly discovered compounds with antibacterial activity.

Spirotetronate compounds. Among novel molecules that were reported since 2011 is maklamicin (Fig. 1), a new spirotetronate-class polyketide. It is a polycyclic compound from the culture extract of endophytic *Micromonospora* sp. GMKU326 isolated in Thailand. Maklamicin showed potent antimicrobial activities against Gram-positive bacteria, including *Micrococcus luteus*, *Bacillus subtilis*, *Bacillus cereus*, *Staphylococcus aureus*, and *Enterococcus faecalis* with MIC values of 0.2, 1.7, 6.5, 13, and 13 μg/ml, respectively, and significantly lower activity against *Candida albicans* (MIC = 50 μg/ml). The compound displayed also moderate cancer cell cytotoxicity (Igarashi et al. 2011).

Another newly discovered spirotetronate antibiotic of polyketide origin is nomimicin, which was isolated from the culture extract of *Actinomadura* sp. TP-A0878. The molecule showed antimicrobial activities against *M. luteus*, *C. albicans* and *Kluyveromyces fragilis* with MIC values of 6.3, 12.5 and 12.5 μg/ml, respectively. Moreover, nomimicin displayed weak cytotoxicity against human cancer cells (Igarashi et al. 2012).

Significant antibacterial and antitumor activities were demonstrated by lobophorin F, a new spirotetronate molecule isolated from *Streptomyces* sp. SCSIO 01127. The MIC values towards *Bacillus thuringiensis*, *S. aureus* and *E. faecalis* equaled 2, 8, 8 μg/ml, respectively (Niu et al. 2011). Interesting bioactivities were demonstrated by secondary metabolites of *Streptomyces* sp. strain MS1 00061 originating from the South China Sea. The three detected secondary metabolites belong to the lobophorin family, and one of them, lobophorin G, was described for the first time. The others, lobophorin A and B, were previously known as anti-inflammatory agents. Together, these three metabolites exhibited a significant anti-BCG (anti-*Mycobacterium bovis*) effect. Furthermore, they exhibited moderate anti-tuberculosis (anti-*Mycobacterium tuberculosis*) properties and diverse activity towards *B. subtilis* (Chen et al. 2013). Another, novel lobophorin H inhibited the growth of *M. tuberculosis*, *B. subtilis* and *S. aureus* and

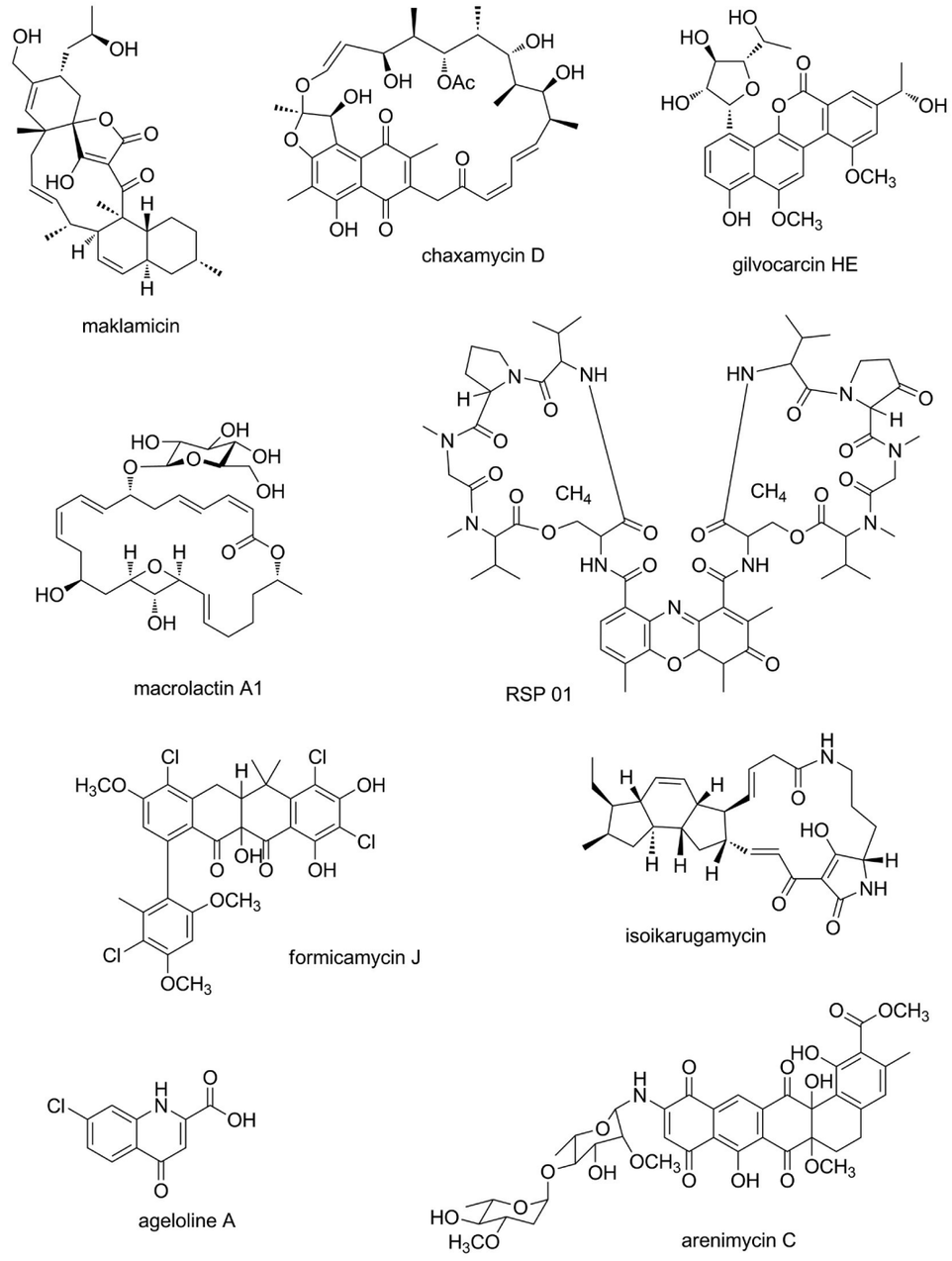

Fig. 1. Chemical structures of compounds with antibacterial activities.

was toxic towards the human CEM-TART cell line (Pan et al. 2013; Lin et al. 2014).

Ansamycin-type compounds. Four new ansamycin-type (aromatic moiety bridged by an aliphatic chain) polyketides, named chaxamycins A-D were isolated from the *Streptomyces* sp. strain C34 from the Chilean hyper-arid Atacama Desert soil. Antibacterial activity assays showed chaxamycin D (Fig. 1) to present the strongest activity against *S. aureus* ATCC 25923 and *Escherichia coli* ATCC 25922 (MIC = 0.05 and 1.21 μg/ml, respectively) as well as against a panel of methicillin-resistant *S. aureus* (MRSA) clinical isolates with MICs ranging from 0.06–0.25 μg/ml (Rateb et al. 2011a). The other chaxamycins (A-C) exhibited also the ability to inhibit the intrinsic ATPase activity of the heat shock protein 90 (Hsp) (41–46% of inhibition at 100 μM).

β-diketones, aromatic ketones. Three new bioactive β-diketones were discovered from the culture broth of *S. asenjonii* KNN 42.f isolated from an extreme hyper-arid Atacama Desert soil in Northern Chile. In addition to thirteen already known substances, three novel compounds – asenjonamides A, B and C, were also purified.

These new active metabolites were grouped in the β-diketone family of polyketides. The highest antibacterial activity was determined for asenjonamide C, which exhibited MIC = 1.8 μg/ml against methicilin-sensitive *S. aureus* (MSSA), MIC = 3.9 μg/ml against *E. faecium* and MIC = 5.4 μg/ml against *E. coli* (Abdelkader et al. 2018).

Moreover, significant antimicrobial properties against *S. aureus*, *B. subtilis*, *E. coli* and *C. albicans* (MICs between 0.25–2.5 μg/ml) were determined for a new polyketide glycoside, gilvocarcin HE (Fig. 1), obtained from the ethyl acetate extract of *Streptomyces* sp. QD01-2 (isolate from soil sample collected at Huiquan Square in China). The compound showed also moderate cytotoxicity against the MCF-7, K562 and P388 cell lines, with IC_{50} values of 36, 39 and 45 μM, respectively. Results prove that the vinyl side chain helps to increase the cytotoxicity and antimicrobial activities of gilvocarcin-type glycosides (Hou et al. 2012).

Two new chloroanthrabenzoxocinones antibiotics – zunyimycins B and C – were isolated from the *Streptomyces* sp. FJS31-2 fermentation broth. The strain was isolated from a soil sample from the Fanjing Mountain of Guizhou Province, China. Zunyimycin C presents good antimicrobial activity toward *S. aureus* ATCC 29213 (MIC = 0.94 μg/ml) and five clinical MRSA isolates (MICs between 3.75–8.14 μg/ml) (Lü et al. 2017).

Tetracenediones. Novel polyketides named formicamycins A-L were discovered as secondary metabolite products of the *S. formicae* KY5 strain isolated from Kenyan ants *Tetraponera penzigi*. Formicamycin J (Fig. 1) was shown to inhibit the growth of the clinically relevant pathogens – MRSA and vancomycin-resistant *Enterococcus faecium* (VRE) with MICs of 0.41 and 0.82 μg/ml, respectively. Other tested formicamycins were less potent (Qin et al. 2017).

Lactones. Application of the "one strain-many compounds" approach on the *Streptomyces* strain using a range of cultivation media resulted in the isolation and identification of several new compounds. These included three new chaxalactins A-C from the rare class of macrolactone polyketides detected together with already known deferroxamine E, hygromycin A, and 5"-dihydrohygromycin A. Novel molecules showed strong activity against Gram-positive bacteria, such as *S. aureus*, *L. monocytogenes*, *B. subtilis* (MICs ranging from 0.2 to 6.3 μg/ml) and weaker activity against Gram-negative strains like *V. parahemolyticus* with MIC of 12.5 μg/ml for chaxalactins A and C and 20 μg/ml for chaxalactin B (Rateb et al. 2011b).

In 2013, Jang and colleagues described a new, promising natural product especially effective towards *Bacillus anthracis* (MIC = 0.03 μg/ml). The compound was designated as anthracimycin and was shown to display antibacterial activity also against *E. faecalis*, *S. pneumoniae*, *H. influenzae*, *Moraxella catarrhalis*, MSSA, MRSA and vancomycin-resistant *S. aureus* with MICs ranging from 0.03 to 4 μg/ml. Anthracimycin was isolated from the *Streptomyces* sp. strain CNH365 collected from marine sediments near Santa Barbara, California, USA. The compound was classified as a polyketide antibiotic and possesses a 14-membered lactone ring and a chemical structure similar to that observed for the macrolide chlorotonil. The mode of action of anthracimycin is based on the inhibition of DNA/RNA synthesis (Jang et al. 2013). Additionally, *in vivo* experiments revealed that anthracimycin was able to protect mice from MRSA-induced mortality in a murine peritonitis model of infection (Hensler et al. 2014).

An actinomycete strain isolated from marine sediment from Chuuk, Federated States of Micronesia, was discovered to produce two new macrolactins – A1 (Fig. 1) and B1 (polyene cyclic macrolactones), and the previously known lauramide diethanolamine. All of them significantly inhibited the growth of Gram-positive (*B. subtilis*, *S. aureus*) and Gram-negative (*E. coli*, *P. aeruginosa*) bacteria with MICs ranging between 0.015 and 0.125 μg/ml. Furthermore, they also displayed potent anti-yeast effects (MIC towards *S. cerevisiae* – 0.125 μg/ml). Unfortunately, activities of these compounds were weaker compared to azithromycin and amphotericin B, which served as positive controls in the trials (Mondol and Shin 2014).

Cruz et al. described the first hyperchlorinated angucyclinones produced by *Actinoallomurus* sp. ID145698. Due to their unique structural features and wide distribution among *Actinoallomurus*, authors have designated these angucyclinones as allocyclinones. These compounds are characterized by an unusual lactone ring and present up to four halogens per molecule, with one congener representing the first natural product containing a trichloromethyl substitution on an aromatic system. The antibacterial activity of four isolated allocyclinones was determined to increase with the number of chlorine substituents on the methyl group. Allocyclinone A has three additional chlorine atoms at carbon C-13. This compound showed the highest antibacterial activity. The MICs were in the range of 0.25–0.5 μg/ml against *S. aureus*, *S. pyogenes* and *E. faecalis,* except for *E. faecium* (MIC = 4 μg/ml). As expected, none of the evaluated, clinically relevant resistance mechanisms affected the activity of the compounds (Cruz et al. 2017).

The first-time reported compound RSP 01 (Fig. 1) is a bicyclic chromopeptide lactone from the actinomycin group. It was found to be biosynthesized together with the already described RSP02 by *Streptomyces* sp. RAB12 isolated from soil samples collected from the garden near the CSIR-Indian Institute of Chemical Technology, Hyderabad, India. Both RSP 01 and 02 revealed a chemical structure similar to actinomycin D. However, RSP 01 has a ketocarbonyl group at the fourth car-

bon of the proline moiety, which is absent in actinomycin D. Results of bioactivity assays suggest that RSP 01 has a higher antimicrobial potential in comparison with actinomycin D. The MICs values for RSP 01 ranged from 0.007 to 0.06 μg/ml against *S. aureus*, *P. aeruginosa*, *S. typhi* and *B. subtilis* (Rathod et al. 2018).

Macrolides are also recognized as lactones. They belong to a class of natural products that consists of a macrocyclic lactone ring (14-membered or more) to which one or more deoxy sugar moiety can be attached. Two 16-membered macrolides, tylosin analogues, were obtained from a wbIA disruption mutant of *Streptomyces ansochromogenes* (wbIA, an Actinobacteria-specific gene controlling major developmental transition). These novel natural products exhibited moderate activity against Gram-positive bacteria, such as: *Streptococcus pyogenes*, *Streptococcus pneumoniae*, *B. subtilis*, *B. cereus* and *S. aureus* with MIC values in the range of 3.53–58.5 μg/ml. It is noteworthy that tylosin derivatives showed stronger bactericidal effects towards *S. pneumoniae* than tylosin itself (Lu et al. 2015).

A novel 48-membered polyol macrolide, named quadoctomycin, was isolated from *Streptomyces* sp. MM168-141F8. It showed potent antibacterial activity against *S. aureus*. The MIC values were evaluated for three MSSA, five MRSA and six *E. faecalis* strains and ranged between 1–2 μg/ml. In turn, quadoctomycin did not show antimicrobial activity toward Gram-negative bacteria (Sawa et al. 2018).

In 2017, Khalil et al. reported on the identification of amycolatopsins A, B and C, the representatives of a rare family of glycosylated polyketide macrolides that are related to the apoptolidin, and novel metabolites of ammocidin structure class derived from the southern Australian soil isolate, *Amycolatopsis* sp. MST-108494. Only amycolatopsins A and C were active towards *M. bovis* and *M. tuberculosis,* reaching IC_{50} values of 0.5–7.0 μg/ml. In turn, all three compounds showed cytotoxicity against mammalian NCIH-460 and SW620 cells (Khalil et al. 2017).

Lactams. *Streptomyces zhaozhouensis* CA-185989 from marine sediment collected near Utonde, Equatorial Guinea was found to produce new bioactive secondary metabolites from the class of polycyclic tetramic acid macrolactams, isoikarugamycin (Fig. 1) and 28-N-methylikarugamycin. These novel compounds demonstrated potent antibacterial and antifungal activities and were shown to be active towards *S. aureus*, *C. albicans* and *A. fumigatus* presenting MICs between 1 and 8 μg/ml (Lacret et al. 2014).

Quinones. Heterologous expression of specific gene clusters (corresponding to two eDNA-derived KSβ sequence tags) in *Streptomyces albus* enabled the isolation of new polyketides, arenimycins C (Fig. 1) and D with potent antibacterial activities. Arenimycins C and D belong to benzo[α]naphthacene quinones and were shown to be active towards MRSA USA300 (MIC = 0.098 and 0.19 μg/ml, respectively) and *B. subtilis* RM125 (MIC = 0.0015 and 0.39 μg/ml, respectively) (Kang and Brady 2014).

Identification of pseudonocardians A-C, new diazaanthraquinone analogs, substantiated the potential of marine actinomycetes as a source of novel drugs. These two compounds derived from *Pseudonocardia* sp. SCSIO 01299 collected from the deep-sea sediment of the South China Sea. The activity profile revealed that pseudonocardians A and B were notably potent against bacterial strains as well as tumor cell lines. The MIC values towards *S. aureus*, *E. faecalis* and *B. thuringensis* ranged between 2–4 μg/ml (Li et al. 2011).

Another group of quinones – pyranonaphthoquinone antibiotics, known as selective inhibitors of the serine-threonine kinase AKT (protein kinase B), has been enlarged in 2015 to include a new member – xiakemycin A. The compound was isolated from the culture broth of *Streptomyces* sp. CC8-201 originating from the soil of a Chinese karst cave. In a panel of antibacterial and antiproliferative assays, xiakemycin A demonstrated activity towards Gram-positive bacteria (*S. aureus*, *S. epidermidis*, *E. faecalis* and *E. faecium*) with MICs ranging from 2 to 16 μg/ml (Jiang et al. 2015).

In 2013, Song et al. reported the isolation of four new sesquiterpenoid naphthoquinones, marfuraquinocins A-D, from the fermentation broth of *Streptomyces niveus* SCSIO 3406 (South China Sea, sediment sample). All compounds were evaluated for their potential antibacterial and cytotoxic properties. Marfuraquinocins A, C and D were active towards *S. aureus* ATCC 29213 with equivalent MICs of 8 μg/ml. Additionally, marfuraquinocins C and D exhibited antibacterial activity against the methicillin-resistant *S. epidermidis* clinical isolate shhs-E1 with the same MIC values (8 μg/ml) (Song et al. 2013).

Xin and colleagues discovered also other quinone derivatives with antibacterial and antitumor properties that is produced by actinomycetes as secondary metabolites. Among them were new modified anthraquinones – fradimycins A and B (capoamycin-type agents) isolated from marine *Streptomyces fradiae* PTZ0025. These two substances were active against *S. aureus* with MICs of 6 and 2 μg/ml, respectively. Moreover, these compounds displayed cytotoxicity towards human cancer cell lines (Xin et al. 2012).

Quinolones. Interesting bioactivity was observed for a newly recognized chlorinated quinolone of actinomycetes origin (*Streptomyces* sp. SBT345) obtained from the Mediterranean sponge, *Agelas oroides*. This novel natural product designated as ageloline A (Fig. 1), showed antichlamydial and antioxidant effects. Ageloline inhibited the growth of *Chlamydia trachomatis*

inclusion with IC_{50} value of 2.1 μg/ml. Moreover, the compound was shown not only to reduce oxidative stress, but also to decrease genomic damage induced by the 4-nitroquinoline-1-oxide, an oxidative stress inducer (Cheng et al. 2016).

Xanthones. Another promising substance with antimicrobial potential is buanmycin, a new pentacyclic xanthone isolated from the culture of a marine *Streptomyces* strain from a tidal mudflat in Buan (Republic of Korea). Buanmycin was shown not only to display strong inhibitory activity against both Gram-positive (*S. aureus*, *B. subtilis*, *K. rhizophila*) and Gram-negative bacteria (*Salmonella enterica*, *P. hauseri*) with MICs 0.42–12.5 μg/ml, but also to inhibit the *S. aureus* sortase A, an enzyme that plays key role in adhesion and host invasion by Gram-positive bacteria (with IC_{50} value of 43.2 μM in comparison to IC_{50} = 104.4 μM for the p-hydroxymercuribenzoic acid, the positive control compound). Additionally, buanmycin exhibited potent cytotoxicity with submicromolar IC_{50} values as well as moderate antifungal activity towards *C. albicans* (MIC = 12.5 μg/ml) (Moon et al. 2014). Four novel xanthones (citreamicin θ A (Fig. 2), citreamicin θ B, citreaglycon A and dehydrocitreaglycon) were obtained by Liu et al. from a marine *Streptomyces caelestis* strain. All these compounds demonstrated potent activity towards *S. haemolyticus*, *S. aureus* and *B. subtilis* with MIC values in the range of 0.25–16 μg/ml. However, citreamicin θ A and citreamicin θ B were definitely more active than the other metabolites, probably due to the five-member nitrogen heterocycle in their structures. Additionally, they also showed cytotoxicity against HeLa cells (Liu, Xu et al. 2012).

Aminocoumarins. The combined approach of phylogenetic and chemical analyses of the *Streptomyces* community from marine sediments of British Columbia in Canada allowed discovering a range of structurally diverse actinomycetes secondary metabolites. Four of them were determined as new novobiocin analogues. Two of them, desmethylnovobiocin and 5-hydroxynovobiocin (Fig. 2) were active against MRSA ATCC 33591 with MIC values of 16 and 8 μg/ml, respectively. Novobiocin is known to target the bacterial gyrase by inhibiting ATP hydrolysis (Maxwell and Lawson 2003). Structure-activity relationships (SAR) studies demonstrated that analogues bearing different substituents at 3"-carbamoyl and 4"-OMe noviose moieties, or a 5-H hydroxybenzoate ring exhibited a dramatic decrease or complete elimination of inhibitory activity against MRSA (Dalisay et al. 2013).

Terpenoids. Three new meroterpenoids – napyradiomycins, together with six already known analogues were isolated as secondary metabolites of the *Streptomyces* sp. strain SCSIO 10428 derived from the Xieyang Island (Beihai, Guangxi province, China). Among them were 4-dehydro-4a-dechloronapyradiomycin A1, 3-dechloro-3-bromonapyradiomycin A1 and 3-chloro-6, 8-dihydroxy-8-α-lapachone, of which the second compound displayed the widest bioactivity range. Antibacterial assays revealed that 3-dechloro-3-bromonapyradiomycin A1 is active towards *S. aureus*, *B. subtilis* and *B. thuringensis* with MIC values at the level of 0.5–1 μg/ml. The compound showed also cytotoxicity against four human cancer cell lines (Wu et al. 2013).

A new secondary metabolite, actinomadurol (Fig. 2), was isolated from rare actinomycete strain, *Actinomadura* KC191. The structure of the compounds revealed a unique 19-norditerpenoid-carbon backbone, which provided a novel scaffold for antibiotic discovery. This molecule displayed significant inhibitory activity towards various bacterial strains, such as *B. subtilis* ATCC 6633, *S. aureus* ATCC 6538p, *K. rhizophila* NBRC 12708, *P. hauseri* NBRC 3851 and *S. enterica* ATCC 14028 with MIC values of 0.39 to 3.12 μg/ml (Shin et al. 2016).

Peptides. A promising scaffold for the treatment of Gram-positive bacterial infections is kocurin (PM181104) (Fig. 2), a new thiazozyl peptide obtained from *Kocuria palustris* F-276,345. This metabolite displays much lower MICs (0.008–0.512 μg/ml) towards *B. subtilis* and drug-resistant strains of *S. aureus*, *E. faecium* or *E. faecalis* than the standard antibiotic, linezolid. The most likely mode of action of this agent is inhibition of bacterial growth by blocking its protein biosynthesis at the translation stage. Furthermore, *in vivo* studies revealed that kocurin protected mice from organ-specific infections or even from systemic infections (Mahajan et al. 2013).

Pargamicins B, C and D are new cyclic peptide metabolites, which were isolated from the fermentation broth of a soil actinomycete strain, *Amycolatopsis* sp. ML1-hF4. The structure of pargamicin A was determined in 2008. Pargamicins are structurally unique cyclic peptides consisting of N-methyl-3-hydroxy valine, 4-hydroxy piperazic acid (4-OH-Pip), sarcosine, phenylalanine, N-hydroxy isoleucine (NOH-Ile) and piperazic acid (Pip). The only structural difference between pargamicins is in the Pip(NOH-Ile) moiety. However, despite this structural similarity, antimicrobial activities of pargamicins showed remarkable differences. Pargamicins A and C exhibited potent antibacterial activity against Gram-positive bacteria, including MRSA and VRE. The MIC values of pargamicin C towards MSSA and MRSA were in the range between 2 and 4 μg/ml, while towards *E. faecium* and *E. faecalis* – in the range from 0.5 to 1 μg/ml. In turn, the antibacterial activity of pargamicin B and D against these bacteria was weaker. Both agents demonstrated MIC values equal 8 μg/ml towards *E. faecium* and *E. faecalis*. When assayed against MSSA and MRSA strains it revealed MICs in the range

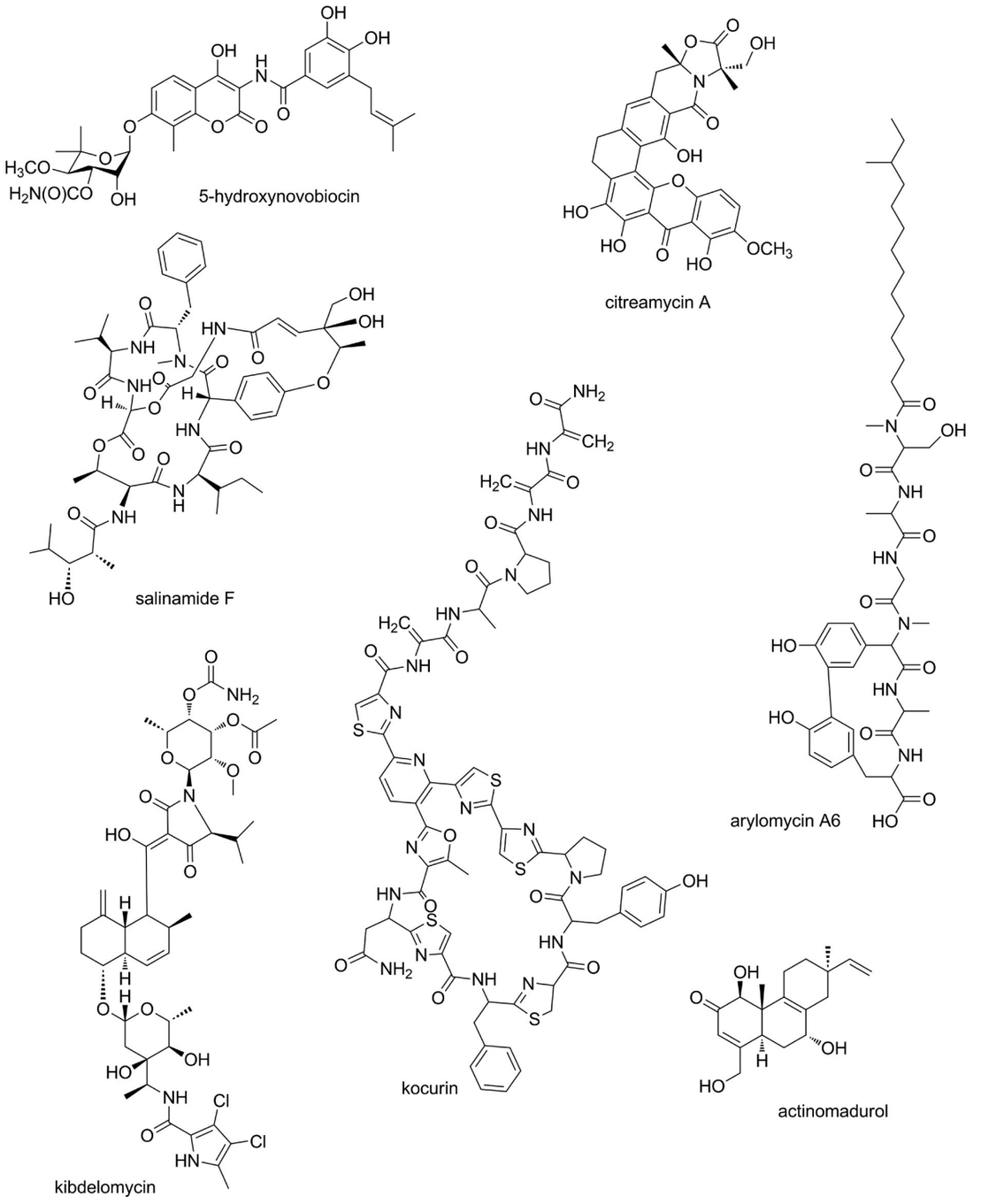

Fig. 2. Chemical structures of compounds with antibacterial activities.

of 8–16 and 32–64 μg/ml for pargamicin B and D, respectively (Hashizume et al. 2017).

Depsipeptides. Investigations of microorganisms from unique environments, such as the sand beach on Jeju, a volcanic island in the Republic of Korea, led to discovery of ohmyungsamycins A and B produced by *Streptomyces* sp. These new cyclic depsipeptides bear unusual amino acid units (N-methyl-4-methoxytrytophan, β-hydroxyphenylalanine, and N,N-dimethylvaline). The two molecules displayed various inhibitory activities against both Gram-positive and Gram-negative bacteria, including *B. subtilis*, *Kocuria rhizophila* and *Proteus hauseri* (MICs = 1.56–49.5 μg/ml). Ohmyungsamycin A is much more potent than ohmyungsamycin B with regard to its cytotoxicity and antibacterial properties (Um et al. 2013).

In 2014, a new bicyclic depsipeptide, salinamide F (Fig. 2), isolated from marine-derived *Streptomyces* sp. CNB-091, was described. The activity profile of salinamide F revealed that it is active towards *E. coli* D21f2tolC, *Haemophilus influenzae*, *E. faecalis* and *Neisseria gonorrhoeae* with MIC values of 0.20, 12.5, 12.5 and 25 μg/ml, respectively. Moreover, it showed significant inhibition of Gram-positive and Gram-negative bacterial RNA polymerase with IC_{50} = 4 μM for *S. aureus* RNAP and 2 μM for *E. coli* RNAP (Hassan et al. 2015).

New natural products discovered by Sun and colleagues, named fijimycins A and C, exhibited activity against three different MRSA strains with MICs in the range of 4–32 μg/ml. These novel etamycin-class depsipeptides were identified from the fermentation broth of *Streptomyces* sp. CNS-575 strain (Nasese, Fiji) (Sun et al. 2011).

Lipopeptides. The use of ultra-performance liquid chromatography coupled with tandem quadrupole and time of flight high-resolution mass spectrometry (UPLC-Q-TOF-HRMS) led to the identification of two lipopeptides from *Streptomyces parvus* HCCB10043. One of them, arylomycin A6 (Fig. 2), was a novel compound and showed antibacterial activity towards *Staphylococcus epidermidis* HCCB20256 with the MIC of 1 μg/ml (Rao et al. 2013).

Other chemical compounds. In 2011, Phillips and coworkers from Merck Research Laboratories, Merck & Co. reported on a compound named kibdelomycin (Fig. 2), which constituted the first structural class of DNA gyrase inhibitors discovered during 60 years of research. This secondary metabolite is a hexacyclic polyketide-peptide hybrid with a dichloropyrrole moiety produced by *Kibdelosporangium* sp. MA7385. The mode of action of kibdelomycin is similar to the coumarin antibiotic – novobiocin, as it targets two subunits of topoisomerase IV and DNA gyrase A. This broad spectrum antibiotic shows activity against MRSA (MIC = 0.5 μg/ml) as well as against *S. pneumoniae* (MIC = 1 μg/ml), *E. faecalis* (MIC = 2 μg/ml) and the Gram-negative pathogen, *Haemophilus influenzae* (MIC = 2 μg/ml) (Phillips et al. 2011). The recent studies performed with clinical strains have revealed potent activity of kibdelomycin against *Acinetobacter baumanii* (MIC = 0.125 μg/ml). There was no cross-resistance observed with other gyrase inhibitors in assays with novobiocin- and ciprofloxacin-resistant *S. aureus* strains (Singh et al. 2015).

Antifungal activity

Among the novel antifungal secondary metabolites produced by actinomycetes is sceliphrolactam. This previously unreported compound was isolated in 2011 from *Streptomyces* sp. associated with the black and yellow mud dauber, *Sceliphron caementarium*, collected in Wisconsin (United States). The structure, which consists of polyunsaturated and polyoxygenated 26-membered macrocyclic lactam is extremely sensitive to light, high temperatures and Lewis acids. Despite its liability, sceliphrolactam demonstrated potent antifungal activity against amphotericin B-resistant *C. albicans* with MIC of 4 μg/ml (Oh et al. 2011).

Other three novel members (15-glycidylfilipin III; 16α, 17α-epoxyfilipin V; 16β, 17β-epoxyfilipin V) of the polyene macrolide class were isolated from the cultures of a soil actinomycete, *S. lavenduligriseus*. Among them, only 15-glycidylfilipin III exhibited strong inhibition of mycelia growth of *C. albicans* with MIC value of 6.25 μg/ml compared to MIC = 3.13 μg/ml for the control, nystatin. The other compounds displayed considerably weaker fungicidal effects (MIC = 200 μg/ml) which ruled out the hypothesis that microbial secondary metabolites with an epoxide function are more toxic or have stronger cytostatic properties than analogs without the epoxide function (Yang et al. 2016).

In 2017, a *Streptomyces* sp. strain was isolated from a soil sample collected from a coal mine at a depth of 20 cm in Nanchang, Jiangxi, People's Republic of China. Six new cyclic octa depsipeptides, enduspeptides A-F, were produced by this strain. The most potent antifungal activities against *C. glabrata* ATCC 90030 with IC_{50} values of 5.33, 1.72 and 8.13 μg/ml were shown by enduspeptides A, B (Fig. 3) and C, respectively (Chen et al. 2017).

Four new Cet1p RNA 5'-triphosphatase inhibitors, designated kribellosides A-D (Fig. 3) were isolated as secondary metabolites of a rare Actinobacteria, *Kribella* MI481-42F6, found in a soil sample collected at Nerima-ku, Tokyo, Japan. These novel alkyl glyceryl ether compounds inhibited the activity of RNA 5'-triphosphatase from *Saccharomyces cerevisiae in vitro* with IC_{50} of 5–8 μM and showed antifungal activity with MICs ranging from 3.12 to 100 μg/ml against *S. cerevisiae* (Igarashi et al. 2017).

Mohangamides A and B were purified from culture broth of marine *Streptomyces* sp. SNM55 obtained from the Mohang mud flat collected in the Buan, Republic of Korea. These unique dilactone-tethered pseudodimeric peptides bear two unusual acyl chains and 14 amino acid residues. The biological activities of these novel compounds were primarily evaluated against *C. albicans* isocitrate lyase (ICL), which is a key enzyme of the glyoxylate cycle and enables microorganisms to grow on acetate, ethanol, or fatty acids in host environments. Mohangamides A and B displayed inhibition against ICL with an IC_{50} value of 4.4 μM and 20.5 μM, respectively. These compounds did not show significant antifungal activity when fungi were fed with glucose. Interestingly,

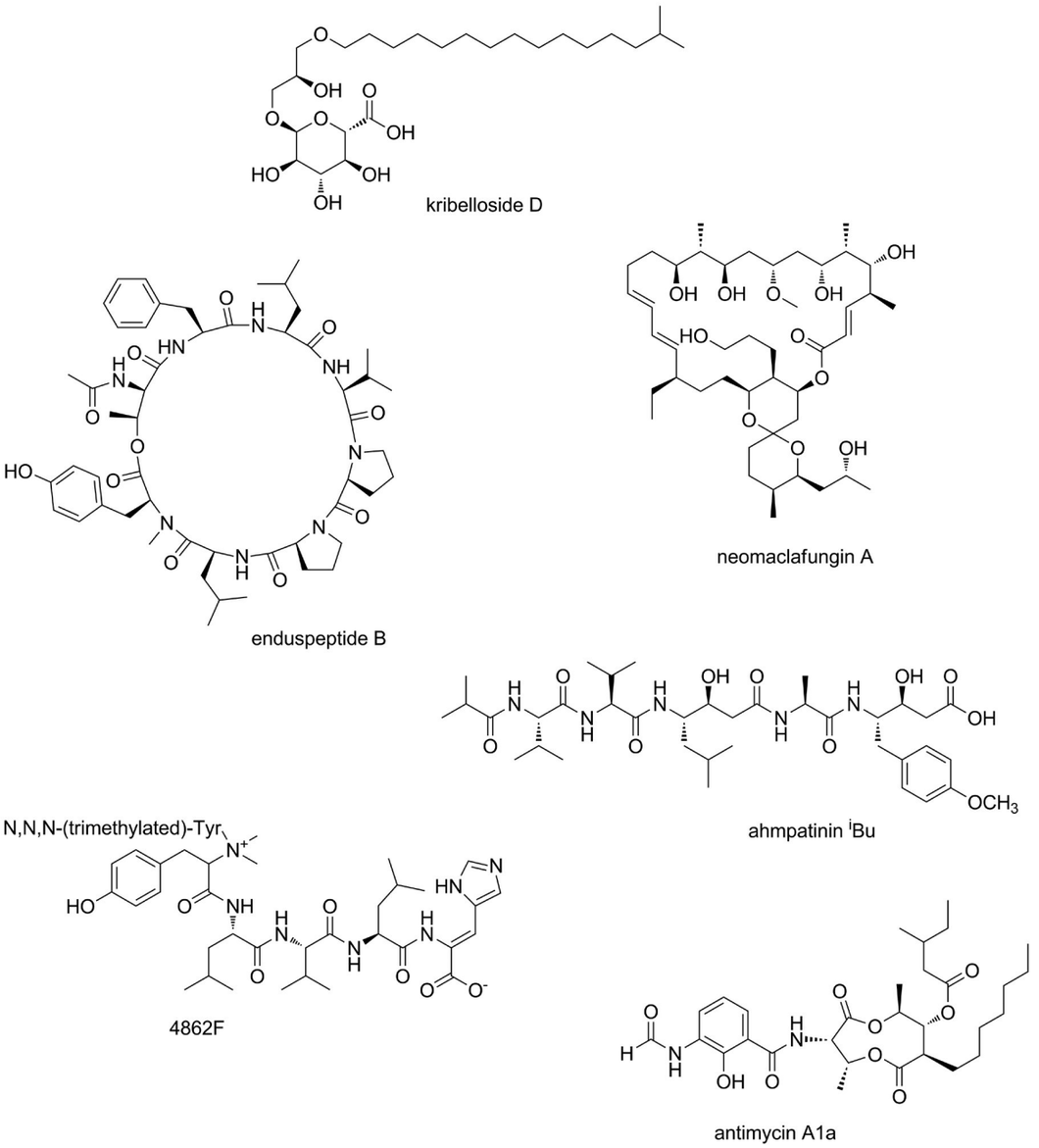

Fig. 3. Chemical structures of compounds with antifungal and antiviral activities.

mohangamide A inhibited *C. albicans* that was grown on 2% sodium acetate (Bae et al. 2015).

Neomaclafungin A (Fig. 3) was the main metabolite from the fermentation broth of *Actinoalloteichus* sp. NPS702 that was isolated from the marine sediment collected from Usa Bay, Kochi Prefecture, Japan. Besides neomaclafungin A, also other neomaclafungins (B-I) were isolated, but in lower concentrations. All novel compounds possess a macrolide ring similar to that of maclafungin, but with different moieties substituted in C-24 and C-33 position. The MIC values for these new neomaclafungins (A-I) against dermatophyte *Trichophyton mentagrophytes* ATCC 9533 were between 1–3 μg/ml (Sato et al. 2012).

Antiviral activity

The study of a marine actinomycetes strain, *Streptomyces kaviengensis*, isolated from the coast of New Ireland (Papua New Guinea) enabled Raveh and coworkers to identify a novel metabolite with significant antiviral activity. The compound, antimycin A1a (Fig. 3), was found to be an antimycin A derivative and shows high potency against the Western equine encephalitis virus with IC_{50} value of less than 4 nM and selectivity index of greater than 550. Analysis of its mechanism of action revealed disruption of mitochondrial electron transport and pyrimidine biosynthesis. Moreover, the previously known antimycin A demonstrated

a broad spectrum activity towards a wide range of RNA viruses, including members of the *Togaviridae*, *Flaviviridae*, *Bunyaviridae*, *Picornaviridae*, and *Paramyxoviridae* families (Raveh et al. 2013).

Promising chemical skeletons with antiviral properties are displayed by xiamycins C-E, produced by *Streptomyces* sp. #HK18 from the topsoil of a Korean solar saltern. New compounds were elucidated as carbazole-bearing indolosesquiterpenoids. Among them, xiamycin D exhibited the strongest effect on porcine epidemic diarrhea virus (PEDV) with replication with value of EC_{50} equaling 0.93 μM (cytotoxicity = 56.03 μM; selectivity index = 60.31). Additionally, the inhibitory activity of xiamycin D was confirmed by quantitative real-time PCR after amplification of fragments of the genes encoding essential structural proteins (GP6 nucleocapsid, GP2 spike, and GP5 membrane) for PEDV replication and by Western blotting of PEDV GP2 spike and GP6 nucleocapsid proteins (Kim et al. 2016).

HIV-1 protease is essential in the life cycle of HIV and has been used as a promising target for AIDS therapy. Several novel potent inhibitors of this protease have been reported recently. In 2013, a new inhibitor of HIV-1 protease, 4862F (Fig. 3) was isolated from the culture broth of *Streptomyces albosporus* I03A-04862. It was elucidated as *N,N,N*-(trimethylated)-Tyr-L-Leu-L-Val-L-Leu-(dehydrated)-His and determined to display inhibitory activity against HIV-1 protease with an IC_{50} value of 15.26 nM (Liu, Gan et al. 2012).

Ahmpatinin ^{i}Bu (Fig. 3) is a linear peptide and a novel pyrrolidine derivative with an unusual amino acid, 4-amino-3-hydroxy-5-(4-methoxyphenyl) pentanoic acid. It was produced by *Streptomyces* sp. CPCC 202950 cultured on sterile soaked rice. Ahmpatinin ^{i}Bu showed significant inhibitory activity against HIV-1 protease resulting in IC_{50} = 1.79 nM (Chen et al. 2018).

Functional diversity of microbial natural products is favorable in regards to exploring for drugs against the Zika virus (ZIKV). The spread of ZIKV into North and South America in 2013 has caused infections of millions of people (Saiz and Martín-Acebes 2017). In 2015, WHO declared a public health emergency due to Zika (Blázquez and Saiz 2016). Lately, a great effort has been put to determine drug candidates directed to viral targets or against cellular targets (host-targeting antivirals). This approach included screening of libraries with different compounds and repurposing drugs already used in clinical practice for other diseases (Bae et al. 2015). Among them, daptomycin (IC_{50} = 1 μM) and nanchangmycin (IC_{50} = 0.1 μM) showed a previously not described anti-ZIKV activity (Barrows et al. 2016; Pascoalino et al. 2016; Rausch et al. 2017). Both of these drugs are bioactive secondary products of *Streptomyces* spp., for which antiviral activity was unrecognized before. Daptomycin is a lipopeptide antibiotic used in the treatment of infections caused by Gram-positive bacteria. It was found in the culture broth of *S. roseosporus*. In turn, nanchangmycin is produced by *S. nanchangensis* and was shown to have insecticidal activity against silkworms and antibacterial activity *in vitro.*

Summary

In this review we report on more than a hundred of natural products of cultured actinomycetes, which exhibit antibacterial, antifungal or antiviral activities. Some of these compounds, besides antimicrobial properties show also cytotoxicity towards different tumor cell lines. The majority of presented agents are novel molecules described between 2011 and April 2018. Only a few of them had a known structure or were synthesized earlier but isolated for the first time from natural sources within these last seven years. Moreover, the majority of described compounds were discovered using a traditional approach of screening for metabolites in the actinobacterial fermentation broth. Most of them inhibited the growth of Gram-positive bacteria, which is in accordance with previous results (Butler et al. 2017). Over 70% of the reported metabolites were produced by various *Streptomyces* sp. strains; the other compounds were produced by the following Actinobacteria: *Amycolatopsis*, *Pseudonocardia, Kibdelosporangium, Actinoalloteichus* (family Pseudonocardiaceae), *Micromonospora* (family Micromonosporaceae), *Actinomadura, Actinoallomurus* (family Thermomonosporaceae), *Actinokineospora* (family Actinosynnemataceae), *Kocuria* (family Micrococcaceae), *Actinomyces* (family Actinomycetaceae), *Kribella* (family Nocardioidaceae) and *Nocardia* (family Nocardiaceae). About 40% of the described metabolites were biosynthesized by species originating from marine ecosystems, about 43% were obtained from terrestrial actinomycetes, while 2% of the metabolites were produced by endophytic strains. Others have unknown origin.

The largest number of novel natural products (53) were produced by bacteria collected from Asia (China, South China Sea, Republic of Korea, Thailand, Japan and India), while bacteria from Australia and Oceania (Australia, Micronesia, Papua New Guinea and Fiji), North America (USA, Canada and Bahamas) and finally from South America (Chile) produced equally 10 new compounds. The other bioactive compounds derived from actinomycetes isolated in Africa and Europe.

Chemical structures of the newly discovered metabolites proved to be quite heterologous. Among them were spirotetronates, ansamycin-type compounds, lactones and lactams, glycosylated lactones – macrolides, quinones, peptides and others. Among all new molecules described here, the best activities displayed

arenimycin C (MICs between 0.0015–0.00985 μg/ml against *B. subtilis* and MRSA), bicyclic chromopeptide lactone RSP 01 (MICs between 0.007–0.06 μg/ml against *S. aureus* and *B. subtilis*) and kocurin (MICs in the range of 0.008–0.512 μg/ml against *S. aureus*, *E. faecium* and *E. faecalis*). Furthermore, quite promising properties were shown for chaxamycin D (MICs in the range of 0.06–0.25 μg/ml against *S. aureus* and *E. coli*) and anthracimycin (MICs in the range of 0.03–4.0 μg/ml against *B. anthracis*, *E. faecalis*, *S. pneumoniae*, *H. influenzae*, *M. catarrhalis* and *S. aureus*). Among the identified antifungal compounds, enduspeptide B, neomaclafungins A-I and kribelloside D exhibited the highest activity towards fungi (MICs 1.72, 1–3 and 3.12 μg/ml, respectively). Ahmpatinin ^{i}Bu, antimycin A1a, and pentapeptide 4862F were found to be the strongest antiviral agents with IC_{50} values of 15.26, < 4 and 1.79 nM, respectively.

All antibacterial, antifungal and antiviral agents presented in this review demonstrate the huge potential of actinomycetes as leader producers of novel bioactive molecules, regarded as promising candidates for clinical evaluation in drug development.

The discovery, development and introduction of a new antibiotic to the pharmaceutical industry are very laborious and complicated processes. The chemical structures of antibiotics, especially those derived from nature are complex. They consist of many stereocenters, rotatable bonds, proton donors and acceptors. Optimization of the molecule structure (its function in biological conditions), scale up of metabolites biosynthesis for industrial purposes as well as long time needed for toxicological and clinical studies makes all this procedures very expensive and time consuming. For these reasons, from an economic point of view, pharmaceutical companies prefer developing drugs for chronic diseases rather than for short-term treatments. One the other hand, rapidly increasing resistance of microorganisms makes contemporary bioactive agents less effective in combating different diseases or useless at all. It is also related with excessive and improper use of antibiotics in medicine, veterinary practice and agriculture. In this context, not to remain helpless in terms of bacterial infections, the discovery of new bioactive compounds is absolutely necessary. Both approaches, traditional way relying on distinct microorganisms isolated from different environments, as well as innovative strategy using the "-omics" technologies together with bioinformatics, are still the valid approaches in searching for novel bioactive agents. Especially, molecules with two modes of action and directed against novel targets are extremely desirable (Ziemska et al. 2013). Another possibilities arise from synthetic or semisynthetic modifications of natural products or searching for novel bioactivities among previously known compounds (such strategies significantly reduce research costs). Finally, a special attention should be placed on the education of the society on the wise and proper use of antibiotics in agriculture as well as in treatment of human and animal diseases.

Acknowledgements

This work was supported by the National Institute of Public Health – National Institute of Hygiene in the frame of project No 17/ZŚ.

Literature

Abdelfattah MS, Arai MA, Ishibashi M. 2016. Bioactive secondary metabolites with unique aromatic and heterocyclic structures obtained from terrestrial actinomycetes species. Chem Pharm Bull. 64:668–675.

Abdelkader MSA, Philippon T, Asenjo JA, Bull AT, Goodfellow M, Ebel R, Jaspars M, Rateb ME. 2018. Asenjonamides A-C, antibacterial metabolites isolated from Streptomyces asenjonii strain KNN 42.f from an extreme-hyper arid Atacama Desert soil. J Antibiot (Tokyo). 71:425–431.

Bae M, Kim H, Moon K, Nam S-J, Shin J, Oh K-B, Oh D-C. 2015. Mohangamides A and B, new dilactone-tethered pseudo-dimeric peptides inhibiting *Candida albicans* isocitrate lyase. Org Lett. 17:712–715.

Baltz RH. 2011. Strain improvement in actinomycetes in the postgenomic era. J Ind Microbiol Biotechnol. 38(6):657–666.

Barka EA, Vatsa P, Sanchez L, Gaveau-Vaillant N, Jacquard C, Klenk H-P, Clément C, Ouhdouch Y, van Wezel GP. 2016. Taxonomy, physiology, and natural products of Actinobacteria. Microbiol Mol Biol Rev. 80:1–43.

Barrows NJ, Campos RK, Powell ST, Prasanth KR, Schott-Lerner G, Soto-Acosta R, Galarza-Muñoz G, McGrath EL, Urrabaz-Garza R, Gao J, et al. 2016. A Screen of FDA-approved drugs for inhibitors of Zika virus infection. Cell Host Microbe. 20:259–270.

Blázquez A-B, Saiz J-C. 2016. Neurological manifestations of Zika virus infection. World J Virol. 5:135.

Bruntner C, Binder T, Pathom-aree W, Goodfellow M, Bull AT, Potterat O, Puder C, Hörer S, Schmid A, Bolek W, et al. 2005. Frigocyclinone, a novel angucyclinone antibiotic produced by a *Streptomyces griseus* strain from Antarctica. J Antibiot (Tokyo). 58:346–349.

Butler MS, Blaskovich MA, Cooper M. 2017. Antibiotics in the clinical pipeline at the end of 2015. J Antibiot (Tokyo). 70:3–24.

Carlson S, Tanouye U, Omarsdottir S, Murphy BT. 2014. Phylum-specific regulation of resistomycin production in a *Streptomyces* sp. via microbial coculture. J Nat Prod. 78(3):381–387.

Chater KF. 2016. Recent advances in understanding Streptomyces. F1000Research. 5:2795.

Chen C, Wang J, Guo H, Hou W, Yang N, Ren B, Liu M, Dai H, Liu X, Song F, Zhang L. 2013. Three antimycobacterial metabolites identified from a marine-derived *Streptomyces* sp. MS100061. Appl Microbiol Biotechnol. 97:3885–3892.

Chen M-H, Chang S-S, Dong B, Yu L-Y, Wu Y-X, Wang R-Z, Jiang W, Gao Z-P, Si S-Y. 2018. Ahmpatinin i Bu, a new HIV-1 protease inhibitor, from Streptomyces sp. CPCC 202950. RSC Adv. 8:5138–5144.

Chen Y, Liu R-H, Li T-X, Huang S-S, Kong L-Y, Yang M-H. 2017. Enduspeptides A-F, six new cyclic depsipeptides from a coal mine derived Streptomyces sp. Tetrahedron. 73:527–531.

Cheng C, Othman EM, Reimer A, Grüne M, Kozjak-Pavlovic V, Stopper H, Hentschel U, Abdelmohsen UR. 2016. Ageloline A, new antioxidant and antichlamydial quinolone from the marine sponge-derived bacterium Streptomyces sp. SBT345. Tetrahedron Lett. 57:2786–2789.

Cruz JCS, Maffioli SI, Bernasconi A, Brunati C, Gaspari E, Sosio M, Wellington E, Donadio S. 2017. Allocyclinones, hyperchlorinated angucyclinones from Actinoallomurus. J Antibiot (Tokyo). 70:73–78.

Cumsille A, Undabarrena A, González V, Claverías F, Rojas C, Cámara B. 2017. Biodiversity of Actinobacteria from the South Pacific and the assessment of Streptomyces chemical diversity with metabolic profiling. Mar Drugs. 15:286.

Dalisay DS, Williams DE, Wang XL, Centko R, Chen J, Raymond J. 2013. Marine sediment-derived Streptomyces bacteria from British Columbia, Canada are a promising microbiota resource for the discovery of antimicrobial natural products. PlosOne. 8:1–14.

Fabricio M. Locatelli K-SG and DU. 2016. Effects of trace metal ions on secondary metabolism and morphological development of streptomycetes. Metallomics. 8:469–480.

Goodfellow M, Busarakam K, Idris H, Labeda DP, Nouioui I, Brown R, Kim B-Y, del Carmen Montero-Calasanz M, Andrews BA, Bull AT. 2017. *Streptomyces asenjonii* sp. nov., isolated from hyper-arid Atacama Desert soils and emended description of *Streptomyces viridosporus* Pridham et al. 1958. Antonie Van Leeuwenhoek. 110:1133–1148.

Guo X, Liu N, Li X, Ding Y, Shang F, Gao Y, Ruan J, Huang Y. 2015. Red soils harbor diverse culturable actinomycetes that are promising sources of novel secondary metabolites. Löffler FE, editor. Appl Environ Microbiol. 81(9):3086–3103.

Hashizume H, Sawa R, Yamashita K, Nishimura Y, Igarashi M. 2017. Structure and antibacterial activities of new cyclic peptide antibiotics, pargamicins B, C and D, from *Amycolatopsis* sp. ML1-hF4. J Antibiot (Tokyo). 70:699–704.

Hassan HM, Degen D, Jang KH, Ebright RH, Fenical W. 2015. Salinamide F, new depsipeptide antibiotic and inhibitor of bacterial RNA polymerase from a marine-derived *Streptomyces* sp. J Antibiot (Tokyo). 68:206–209.

Hensler ME, Jang KH, Thienphrapa W, Vuong L, Tran DN, Soubih E, Lin L, Haste NM, Cunningham ML, Kwan BP, et al. 2014. Anthracimycin activity against contemporary methicillin-resistant *Staphylococcus aureus*. J Antibiot (Tokyo). 67:549–553.

Hou J, Liu P, Qu H, Fu P, Wang Y, Wang Z, Li Y, Teng X, Zhu W. 2012. Gilvocarcin HE: a new polyketide glycoside from *Streptomyces* sp. J Antibiot (Tokyo). 65:523–526.

Idris H, Nouioui I, Asenjo JA, Bull AT, Goodfellow M. 2017. *Lentzea chajnantorensis* sp. nov., an actinobacterium from a very high altitude Cerro Chajnantor gravel soil in northern Chile. Antonie Van Leeuwenhoek. 110:795–802.

Igarashi Y, Iida T, Oku N, Watanabe H, Furihata K, Miyanouchi K. 2012. Nomimicin, a new spirotetronate-class polyketide from an actinomycete of the genus Actinomadura. J Antibiot (Tokyo). 65:355–359.

Igarashi Y, Ogura H, Furihata K, Oku N, Indananda C, Thamchaipenet A. 2011. Maklamicin, an antibacterial polyketide from an endophytic *Micromonospora* sp. J Nat Prod. 74(4):670–674.

Igarashi M, Sawa R, Yamasaki M, Hayashi C, Umekita M, Hatano M, Fujiwara T, Mizumoto K, Nomoto A. 2017. Kribellosides, novel RNA 5'-triphosphatase inhibitors from the rare actinomycete *Kribbella* sp. MI481-42F6. J Antibiot (Tokyo). 70:582–589.

Jang KH, Nam S-J, Locke JB, Kauffman CA, Beatty DS, Paul LA, Fenical W. 2013. Anthracimycin, a potent anthrax antibiotic from a marine-derived Actinomycete. Angew Chemie Int Ed. 52: 7822–7824.

Jiang Z, Guo L, Chen C, Liu S, Zhang L, Dai S, He Q, You X, Hu X, Tuo L, et al. 2015. Xiakemycin A, a novel pyranonaphthoquinone antibiotic, produced by the *Streptomyces* sp. CC8-201 from the soil of a karst cave. J Antibiot (Tokyo). 68:771–774.

Kang H, Brady SF. 2014. Mining soil metagenomes to better understand the evolution of natural product structural diversity: pentangular polyphenols as a case study. J Am Chem Soc. 136(52):18111–18119.

Khalil ZG, Salim AA, Vuong D, Crombie A, Lacey E, Blumenthal A, Capon RJ. 2017. Amycolatopsins A-C: antimycobacterial glycosylated polyketide macrolides from the Australian soil Amycolatopsis sp. MST-108494. J Antibiot (Tokyo). 70:1097–1103.

Kim S-H, Ha T-K-Q, Oh WK, Shin J, Oh D-C. 2016. Antiviral indolosesquiterpenoid xiamycins c-e from a halophilic actinomycete. J Nat Prod. 79:51–58.

Komaki H, Ichikawa N, Oguchi A, Hamada M, Tamura T. 2015. Genome-based analysis of non-ribosomal peptide synthetase and type-I polyketide synthase gene clusters in all type strains of the genus Herbidospora. BMC Res Notes. 8:548.

Lacret R, Oves-Costales D, Gómez C, Díaz C, de la Cruz M, Pérez-Victoria I, Vicente F, Genilloud O, Reyes F. 2014. New ikarugamycin derivatives with antifungal and antibacterial properties from *Streptomyces zhaozhouensis*. Mar Drugs. 13:128–140.

Latha S, Sivaranjani G, Dhanasekaran D. 2017. Response surface methodology : A non- conventional statistical tool to maximize the throughput of *Streptomyces* species biomass and their bioactive metabolites. Crit Rev Microbiol. 43(5):567–582.

Lee L-H, Cheah Y-K, Mohd Sidik S, Ab Mutalib N-S, Tang Y-L, Lin H-P, Hong K. 2012. Molecular characterization of Antarctic actinobacteria and screening for antimicrobial metabolite production. World J Microbiol Biotechnol. 28:2125–2137.

Li S, Tian X, Niu S, Zhang W, Chen Y, Zhang H, Yang X, Zhang W, Li W, Zhang S, et al. 2011. Pseudonocardians A-C, new diazaanthraquinone derivatives from a deap-sea actinomycete *Pseudonocardia* sp. SCSIO 01299. Mar Drugs. 9:1428–1439.

Lin Z, Koch M, Pond CD, Mabeza G, Seronay RA, Concepcion GP, Barrows LR, Olivera BM, Schmidt EW. 2014. Structure and activity of lobophorins from a turrid mollusk-associated *Streptomyces* sp. J Antibiot (Tokyo). 67:121–126.

Liu L-L, Xu Y, Han Z, Li Y-X, Lu L, Lai P-Y, Zhong J-L, Guo X-R, Zhang X-X, Qian P-Y. 2012. Four new antibacterial xanthones from the marine-derived actinomycetes *Streptomyces caelestis*. Mar Drugs. 10:2571–2583.

Liu X, Gan M, Dong B, Zhang T, Li Y, Zhang Y, Fan X, Wu Y, Bai S, Chen M, et al. 2012. 4862F, a new inhibitor of HIV-1 protease, from the culture of *Streptomyces* I03A-04862. Molecules. 18:236–243.

Lu C, Liao G, Zhang J, Tan H. 2015. Identification of novel tylosin analogues generated by a wblA disruption mutant of *Streptomyces ansochromogenes*. Microb Cell Fact. 14:173.

Lü Y, Shao M, Wang Y, Qian S, Wang M, Wang Y, Li X, Bao Y, Deng C, Yue C, et al. 2017. Zunyimycins B and C, new chloroanthrabenzoxocinones antibiotics against Methicillin-Resistant *Staphylococcus aureus* and Enterococci from *Streptomyces* sp. FJS31-2. Molecules. 22:251.

Mahajan G, Thomas B, Parab R, Patel ZE, Kuldharan S, Yemparala V, Mishra PD, Ranadive P, D'Souza L, Pari K, Sivaramkrishnan H. 2013. *In vitro* and *in vivo* activities of antibiotic PM181104. Antimicrob Agents Chemother. 57:5315–5319.

Manivasagan P, Venkatesan J, Sivakumar K, Kim S. 2013. Pharmaceutically active secondary metabolites of marine actinobacteria. Microbiol Res. 169(4):262–78.

Maxwell A, Lawson D. 2003. The ATP-binding site of type II topoisomerases as a target for antibacterial drugs. Curr Top Med Chem. 3:283–303.

Medema MH, Blin K, Cimermancic P, de Jager V, Zakrzewski P, Fischbach MA, Weber T, Takano E, Breitling R. 2011. antiSMASH: rapid identification, annotation and analysis of secondary metabolite biosynthesis gene clusters in bacterial and fungal genome sequences. Nucleic Acids Res. 39:W339–W346.

Medema MH, Fischbach MA. 2015. Computational approaches to natural product discovery. Nat Chem Biol. 11:639–648.

Mondol M, Shin H. 2014. Antibacterial and antiyeast compounds from marine-derived bacteria. Mar Drugs. 12:2913–2921.

Moon K, Chung B, Shin Y, Rheingold AL, Moore CE, Park SJ, Park S, Lee SK, Oh K, Shin J. 2014. Pentacyclic antibiotics from a tidal mud flat-derived actinomycete. J Nat Prod. 78(3):524–529.

Newman DJ, Cragg GM. 2016. Natural products as sources of new drugs from 1981 to 2014. J Nat Prod. 79:629–661.

Niu S, Li S, Chen Y, Tian X, Zhang H, Zhang G, Zhang W, Yang X, Zhang S, Ju J, Zhang C. 2011. Lobophorins E and F, new spirotetronate antibiotics from a South China Sea-derived *Streptomyces* sp. SCSIO 01127. J Antibiot (Tokyo). 64:711–716.

O'Neill J. 2016. Tackling drug-resistant infections globally: final report and recommandations [Internet]. London (UK): The review on antimicrobial resistance; [cited 2018 May 8]. Available from https://amr-review.org/sites/default/files/160525_Final%20paper_with%20cover.pdf.

Oh D, Poulsen M, Currie CR, Clardy J. 2011. Sceliphrolactam, a polyene macrocyclic lactam from a wasp-associated. Org Lett. 13:15–18.

Onaka H. 2017. Novel antibiotic screening methods to awaken silent or cryptic secondary metabolic pathways in actinomycetes. J Antibiot (Tokyo). 70(8):865–870.

Pan H-Q, Zhang S-Y, Wang N, Li Z-L, Hua H-M, Hu J-C, Wang S-J. 2013. New spirotetronate antibiotics, Lobophorins H and I, from a South China sea-derived *Streptomyces* sp. 12A35. Mar Drugs. 11:3891–3901.

Pascoalino BS, Courtemanche G, Cordeiro MT, Gil LHVG, Freitas-Junior L. 2016. Zika antiviral chemotherapy: identification of drugs and promising starting points for drug discovery from an FDA-approved library. F1000Research. 5:2523.

Paulus C, Rebets Y, Tokovenko B, Nadmid S, Terekhova LP, Myronovskyi M, Zotchev SB, Rückert C, Braig S, Zahler S, et al. 2017. New natural products identified by combined genomics-metabolomics profiling of marine *Streptomyces* sp. MP131–18. Sci Rep. 7:42382.

Phillips JW, Goetz MA, Smith SK, Zink DL, Polishook J, Onishi R, Salowe S, Wiltsie J, Allocco J, Sigmund J, et al. 2011. Discovery of kibdelomycin, a potent new class of bacterial type II topoisomerase inhibitor by chemical-genetic profiling in *Staphylococcus aureus*. Chem Biol. 18:955–965.

Qin Z, Munnoch JT, Devine R, Holmes NA, Seipke RF, Wilkinson KA, Wilkinson B, Hutchings MI. 2017. Formicamycins, antibacterial polyketides produced by *Streptomyces formicae* isolated from African Tetraponera plant-ants. Chem Sci. 8:3218–3227.

Rajnisz A, Guśpiel A, Postek M, Ziemska J, Laskowska A, Rabczenko D, Solecka J. 2016. Characterization and optimization of biosynthesis of bioactive secondary metabolites produced by *Streptomyces* sp. 8812. Pol J Microbiol. 65:51–61.

Rao M, Wei W, Ge M, Chen D, Sheng X. 2013. A new antibacterial lipopeptide found by UPLC-MS from an actinomycete *Streptomyces* sp. HCCB10043. Nat Prod Res. 27:2190–2195.

Rateb ME, Houssen WE, Arnold M, Abdelrahman MH, Deng H, Harrison WTA, Okoro CK, Asenjo JA, Andrews BA, Ferguson G, et al. 2011a. Chaxamycins A-D, bioactive ansamycins from a hyperarid desert *Streptomyces* sp. J Nat Prod. 74(6):1491–1499.

Rateb ME, Houssen WE, Harrison WTA, Deng H, Okoro CK, Asenjo JA, Andrews BA, Bull AT, Goodfellow M, Ebel R, Jaspars M. 2011b. Diverse metabolic profiles of a Streptomyces strain isolated from a hyper-arid environment. J Nat Prod. 74(9):1965–1971.

Rathod BB, Korasapati R, Sripadi P, Reddy Shetty P. 2018. Novel actinomycin group compound from newly isolated *Streptomyces* sp. RAB12: isolation, characterization, and evaluation of antimicrobial potential. Appl Microbiol Biotechnol. 102:1241–1250.

Rathore SS, Ramamurthy V, Allen S, Selva Ganesan S, Ramakrishnan J. 2016. Novel approach of adaptive laboratory evolution: triggers defense molecules in *Streptomyces* sp. against targeted pathogen. RSC Adv. 6:96250–96262.

Rausch K, Hackett BA, Weinbren NL, Reeder SM, Sadovsky Y, Hunter CA, Schultz DC, Coyne CB, Cherry S. 2017. Screening bioactives reveals nanchangmycin as a broad spectrum antiviral active against Zika virus. Cell Rep. 18:804–815.

Raveh A, Delekta PC, Dobry CJ, Peng W, Schultz PJ, Blakely PK, Tai AW, Matainaho T, Irani DN, Sherman DH, Miller DJ. 2013. Discovery of potent broad spectrum antivirals derived from marine actinobacteria. Ianora A, editor. PLoS One. 8:e82318.

Riquelme C, Dapkevicius MDE, Miller AZ, Charlop-Powers Z, Brady S, Mason C, Cheeptham N. 2016. Biotechnological potential of Actinobacteria from Canadian and Azorean volcanic caves. Appl Microbiol Biotechnol. 101(2):843–857.

Saiz J-C, Martín-Acebes MA. 2017. The race to find antivirals for Zika virus. Antimicrob Agents Chemother. 61:e00411–17.

Sato S, Iwata F, Yamada S, Katayama M. 2012. Neomaclafungins A-I: Oligomycin-class macrolides from a marine-derived Actinomycete. J Nat Prod. 75:1974–1982.

Sawa R, Kubota Y, Umekita M, Hatano M, Hayashi C, Igarashi M. 2018. Quadoctomycin, a 48-membered macrolide antibiotic from *Streptomyces* sp. MM168-141F8. J Antibiot (Tokyo). 71:91–96.

Shin B, Kim B, Cho E, Oh K, Shin J, Goodfellow M. 2016. Actinomadurol, an antibacterial norditerpenoid from a rare actinomycete, *Actinomadura* sp. KC 191. J Nat Prod. 79(7):1886–1890.

Singh B, Gupta V, Passari A. 2018. New and future developments in microbial biotechnology and bioengineering. Actinobacteria: diversity and biotechnological applications. Amsterdam, Oxford, Cambridge: Elsevier.

Singh SB, Dayananth P, Balibar CJ, Garlisi CG, Lu J, Kishii R, Takei M, Fukuda Y, Ha S, Young K. 2015. Kibdelomycin is a bactericidal broad-spectrum aerobic antibacterial agent. Antimicrob Agents Chemother. 59:3474–3481.

Solecka J, Zajko J, Postek M, Rajnisz A. 2012. Biologically active secondary metabolites from Actinomycetes. Cent Eur J Biol. 7:373–390.

Solecka J, Ziemska J, Rajnisz A, Laskowska A, Guśpiel A. 2013. Promieniowce – Występowanie i wytwarzanie związków biologicznie czynnych. Postep Mikrobiol. 52:83–91.

Song Y, Huang H, Chen Y, Ding J, Zhang Y, Sun A, Zhang W, Ju J. 2013. Cytotoxic and Antibacterial marfuraquinocins from the deep South China Sea-derived *Streptomyces niveus* SCSIO 3406. J Nat Prod. 76(12):2263–2268.

Sun P, Maloney KN, Nam S-J, Haste NM, Raju R, Aalbersberg W, Jensen PR, Nizet V, Hensler ME, Fenical W. 2011. Fijimycins A-C, three antibacterial etamycin-class depsipeptides from a marine-derived *Streptomyces* sp. Bioorg Med Chem. 19:6557–6562.

Thong WL, Shin-ya K, Nishiyama M, Kuzuyama T. 2015. Methylbenzene-containing polyketides from a Streptomyces that spontaneously acquired rifampicin resistance: structural elucidation and biosynthesis. J Nat Prod. 79(4):857–864.

Um S, Choi TJ, Kim H, Kim BY, Kim S, Lee SK, Oh K, Shin J, Oh D. 2013. Ohmyungsamycins A and B: cytotoxic and antimicrobial cyclic peptides produced by *Streptomyces* sp. from a Volcanic Island. J Org Chem. 78(24):12321–12329.

WHO. 2014. Antimicrobial resistance: global report on surveillance [Internet]. Geneva (Switzerland): World Health Organization;

[cited 2018 May 8]. Available from http://apps.who.int/iris/bitstream/10665/112642/1/9789241564748_eng.pdf?ua=1

WHO. 2017. Global priority list of antibiotic-resistant bacteria to guide research, discovery, and development of new antibiotics [Internet]. Geneva (Switzerland): World Health Organization; [cited 2018 May 8]. Available from http://www.who.int/medicines/publications/WHO-PPL-Short_Summary_25Feb-ET_NM_WHO.pdf?ua=1

Wink J, Mohammadipanah F, Hamedi J, editors. 2017. Biology and Biotechnology of Actinobacteria. Cham (Switzerland): Springer Nature.

Wu Z, Li S, Li J, Chen Y, Saurav K, Zhang Q, Zhang H, Zhang W, Zhang W, Zhang S, Zhang C. 2013. Antibacterial and cytotoxic new napyradiomycins from the marine-derived *Streptomyces* sp. SCSIO 10428. Mar Drugs. 11:2113–2125.

Xin W, Ye X, Yu S, Lian X-Y, Zhang Z. 2012. New capoamycin-type antibiotics and polyene acids from marine *Streptomyces fradiae* PTZ0025. Mar Drugs. 10:2388–2402.

Xu J, Gu K, Zhang D-J, Li Y-G, Tian L. 2017. Ghanamycins A and B, two novel γ-butyrolactones from marine-derived *Streptomyces ghanaensis* TXC6-16. J Antibiot (Tokyo). 70:733–736.

Yang J, Yang Z, Yin Y, Rao M, Liang Y, Ge M. 2016. Three novel polyene macrolides isolated from cultures of *Streptomyces lavenduligriseus*. J Antibiot (Tokyo). 69:62–65.

Yu L, Trujillo ME, Miyanaga S, Saiki I, Igarashi Y. 2014. Campechic acids A and B: anti-invasive polyether polyketides from a soil-derived Streptomyces. J Nat Prod. 77(4):976–982.

Zhang H, Wang H, Wang Y, Cui H, Xie Z, Pu Y, Pei S, Li F, Qin S. 2012. Genomic sequence-based discovery of novel angucyclinone antibiotics from marine *Streptomyces* sp. W007. FEMS Microbiol Lett. 332(2):105–112.

Ziemska J, Rajnisz A, Solecka J. 2013. New perspectives on antibacterial drug research. Cent Eur J Biol. 8:943–957.

Zotchev SB. 2012. Marine actinomycetes as an emerging resource for the drug development pipelines. J Biotechnol. 158:168–175.

Polish Journal of Microbiology
2018, Vol. 67, No 3, 273–281
https://doi.org/10.21307/pjm-2018-032

ORIGINAL PAPER

Exopolysaccharides Produced by *Lactobacillus rhamnosus* KL 53A and *Lactobacillus casei* Fyos Affect Their Adhesion to Enterocytes

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Submitted 1 February 2017, revised 28 February 2018, accepted 20 April 2018

Abstract

Probiotics promote and help to maintain beneficial microbiota composition of the gastrointestinal tract ecosystem and have a positive impact on the host's health. Production of exopolysaccharides is an important feature of probiotic lactobacilli. It increases the chance of their survival in the gastrointestinal tract and promotes adhesion to the epithelium; therefore, exopolysaccharides are important for the process of colonization. Two lactic acid bacteria strains were used in this study: *Lactobacillus rhamnosus* KL 53A and *Lactobacillus casei* Fyos. Exopolysaccharides were isolated from bacterial cells and their monosaccharide composition was examined using liquid chromatography. The influence of exopolysaccharides on lactobacilli adhesion to enterocytes was studied after deglycosylation of the bacterial cells and incubation with the selected intestinal microbiota strains that metabolize polysaccharides – *Faecalibacterium prausnitzii* DSM 17677 and *Blautia luti* DSM 14534. Both deglycosylation and incubation with polysaccharide metabolizing strains influenced the ability of probiotic strains to adhere to enterocytes. Enzymatic deglycosylation decreased adhesion efficiency of *L. rhamnosus* KL 53A; however, co-incubation of both lactobacillus strains with *F. prausnitzii* DSM 17677 resulted in an increase of their adhesion efficiency. Exopolysaccharides are important adhesins of *Lactobacillus* spp. that influence their ability to colonize gut epithelium. Other members of gut microbiota can modify the adhesion property *in situ*; therefore the composition and metabolic state of commensal bacteria may influence their probiotic action.

Key words: bacteria, adhesion, deglycosylation, polysaccharides, Caco-2 cells

Introduction

Exopolysaccharides (EPS) are extracellular biopolymers that are produced by many species of microorganisms, including *Lactobacillus* spp. (Ruas-Madiedo and de los Reyes-Gavilán 2005). They may remain attached to the bacterial envelope or be secreted into the environment in the form of mucus (Ruas-Madiedo et al. 2006).

In terms of structure, two main groups of EPS produced by bacteria are distinguished: homo- and heteropolysaccharides. Homopolysaccharides consist of one type of monosaccharide moiety, typically glucose or fructose, and have a simple structure. Differences between their various types are related to the characteristics of the primary structure, such as the type of monomer and bonds between the monomer subunits. Heteropolysaccharides have a more complex structure. They are composed of monosaccharides belonging to at least two different types. The structure of individual heteropolysaccharides may vary considerably. The common feature for most of them is the presence of glucose, rhamnose and galactose, found at different ratios (Welman and Maddox 2003).

There are two known pathways of EPS synthesis in lactic acid bacteria, i.e. the extracellular glucosyltransferase or fructosyltransferase (GT) dependent pathway, and the Wzy/Wzx-dependent pathway (Ryan et al. 2015). The substrate used for the synthesis of homopolysaccharides via the GT dependent pathway is sucrose or another oligosaccharide containing fructose for fructan synthesis (Galle and Arendt, 2014), and the energy used in this process derives from its hydrolysis. Glucosyltransferases carry out the synthesis of glucans, while fructosyltransferases are involved in the formation of fructans (Korakli et al. 2003). Many different types of GT are encoded in the genomes of lactobacilli and only a small part of them have been biochemically characterized (van Hijum et al. 2006). The GT pathway is a relatively simple enzymatic process, which requires low energy inputs (Ryan et al. 2015).

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The Wzy/Wzx-dependent pathway is a more complex enzymatic process, with several enzymes and regulatory proteins involved in heteropolysaccharides synthesis and secretion. It initially takes place in the cytoplasm, where repeating units of heteropolysaccharides are assembled by the glucosyltransferases. The Wzx flippase translocates the repeated units into the periplasm, where polymerization by the Wzy polymerase takes place. In the last stage, polysaccharide is transported outside the cell by the OPX protein (Schmid et al. 2015).

Most stages of exopolysaccharide biosynthesis occur in the cytoplasm (Welman and Maddox 2003). Polymerization or EPS secretion into the environment takes place within the cell membrane. In some cases (e.g. dextran, levan) the synthesis of EPS can be entirely extracellular (Schmid et al. 2015).

The amount and type of synthesized heteropolysaccharides depend on the environment, in which the strain grows (De Vuyst and Degeest 1999). Depending on the composition of the medium, temperature, pH and oxygen content, lactobacilli can produce exopolysaccharides of different sugar monomers, interconnected by different types of bonds (De Vuyst and Degeest 1999; Furukawa et al. 2000).

The ability of lactic acid bacteria to produce EPS is commonly used in the dairy industry to improve texture and to stabilize the products (Patel and Prajapati 2013). The physiological role of EPS produced by lactic acid bacteria remains, however, little known. EPS affect the surface properties of bacterial cells. EPS play an important role in adhesion and colonization of the gastrointestinal tract by probiotics. They also promote the formation of the biofilm that facilitates colonization of the epithelium and increases the chance of bacterial survival in the gastrointestinal tract (Lebeer et al. 2009; Stack et al. 2010). EPS enhance the hydrophobicity of the bacterial cell surface, increasing the ability of bacteria to bind to the intestinal mucosa (Sun et al. 2007), and likely participate in enterocyte adhesion as ligands for epithelial surface receptors (Ruas-Madiedo et al. 2006). There are also reports that EPS protect against phage attack and phagocytosis (Gopal and Crow 1993; Comstock and Kasper 2006).

Exopolysaccharides may also act as prebiotics, i.e. non-digestible food ingredients that are beneficial for the host organism by selectively stimulation of growth and/or activity of one or more species of bacteria colonizing the gut. It has been shown that certain bacteria belonging to *Firmicutes* or *Bacteroides* in the intestinal ecosystem of humans metabolize the EPS produced by *Lactobacillus* and *Bifidobacterium* species (Rios-Covian et al. 2016). The interaction between the different species of microorganisms present in the intestines is one of the factors that influence the composition of the intestinal ecosystem (Wexler et al. 2016). This also applies to probiotic bacteria delivered to the digestive tract (O'Toole et al. 2008). Their beneficial effect on the host organism can be modified by species of native microbiota, for example by limiting their adhesion to the intestinal epithelium and thereby reducing their time within the intestine.

The aim of our work was to determine the role of EPS on adhesion of *L. rhamnosus* KL 53A and *L. casei* Fyos to the gut epithelium and to investigate whether intestinal commensal microorganisms have the ability to metabolize EPS synthesized by the probiotics, therefore *in situ* influencing their properties. Two strains *F. prausnitzii* DSM 17677 and *B. luti* DSM 14534, the representatives of the species that are common in the intestinal microbiota, have been selected for our study.

Experimental

Materials and Methods

Human and bacterial cells. Two lactic acid bacteria strains were used in this study: *L. rhamnosus* KL 53A (derived from a dietary supplement Dicoflor, Vitis Pharma, Warsaw, Poland) and *L. casei* Fyos (derived from a probiotic drink). Lactic acid were cultured in the MRS medium (Biocorp, Warsaw, Poland) at 37°C in an anaerobic jar. For the adhesion tests, the Caco-2 cell line was used (obtained from European Collection of Cell Cultures). The Caco-2 cell line derived from a colorectal cancer shows morphology and functional features of normal small intestine cells in post-confluent culture. Caco-2 cells are widely recognized as a substitute for the human intestine epithelial cells and are used to study.

Adhesion and invasion of intestinal pathogens. Caco-2 cells were cultured in Dulbecco's modified Eagle's medium (Lonza, Belgium) supplemented with 10% (v/v) heat-inactivated calf serum (Lonza, Belgium) and 1% (v/v) nonessential amino acids (Lonza, Belgium). The cells were grown in a humidified atmosphere of 10% CO_2 at 37°C. To examine the influence of the bacterial strains that metabolize exopolysaccharides on the adhesion of lactobacilli to enterocytes, two strains of bacteria that occur in the human intestines were used: *B. luti* DSM 14534 and *F. prausnitzii* DSM 17677 (Touyama et al. 2015; Rios-Covian et al. 2016). Strains were derived from DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany). Bacteria were grown in Anaerobe Basal Broth (Oxoid, Basingstoke, United Kingdom), at 37°C in an anaerobic jar.

Isolation, purification and quantification of exopolysaccharides from lactic acid bacteria. Bacterial cells were grown in MRS broth at 37°C in anaerobic

conditions for 24 h. After this time, 100 µl of bacterial culture was plated on MRS agar and incubated at 37°C in anaerobic conditions for 72 h. Bacterial biomass was washed from the plate with deionized, sterile water. Then, one volume of 2 M NaOH was added to bacterial suspension. After incubation overnight in room temperature (22°C), sample was centrifuged (2800 × g, 20 min). Two volumes of ice-cold ethanol were added to the supernatant and incubated for 48 h at 4°C until the EPS precipitated. At the next step, samples were centrifuged (2800 × g, 30 min), supernatant was discarded and EPS were resuspended in sterile water and dialyzed for 72 h using the D-tube dialyzer Mega, 6–8 kDa (Merck, Warsaw, Poland). Then the samples were frozen at –80°C. The monomer composition of EPS was analyzed using HPLC (Rezex RSO-Oligosaccharide Ag^+ 200 × 10.0 mm column with 60 × 10.0 mm precolumn, a RID refractometric detector, water as the mobile phase, column temperature: 80°C, flow rate: 0.3 ml/min, injection volume: 10 µl, pressure: 24 bar, run time: 1 h). Before HPLC analysis samples were hydrolyzed using acidic hydrolysis. A volume of 1 ml of 25% (v/v) sulfuric acid was added per 100 mg of sample and incubated for 12 h at 4°C. Then 1 ml of 70% sulfuric acid (v/v) was added and the sample was incubated in a water bath at 55°C for 2 hours. After this time samples were cooled at room temperature and diluted with 10 ml of distilled water. The next step consisted of incubation in a water bath at 90°C for 1 h. In the last stage samples were cooled at room temperature, neutralized by adding 500 mg of calcium carbonate and filtered using a syringe filter with a 0.2 µm pore size hydrophilic PTFE membrane.

Deglycosylation of bacterial cells. Enzymatic deglycosylation of bacterial cell surface was made using the Protein Deglycosylation Mix (Bio Labs, Ipswich, Massachusetts, USA) (Table I) according to the instructions for non-denaturing reaction conditions. Before deglycosylation, the bacterial cells were labelled with methyl-[^{3}H]-thymidine (5 µl/ml of broth, 60–90 Ci/mmol, 1 mCi/ml) as described previously (Schmidt et al. 2010) and inactivated with 4% formaldehyde, as described in our previous paper (Markowicz et al. 2016) to prevent EPS synthesis during and after the deglycosylation step. Briefly, the bacterial cells were grown in MRS broth at 37°C under anaerobic conditions for 18–20 h. After this time, bacteria were washed twice with PBS and incubated in 4% formaldehyde in PBS for 24 h at room temperature. After inactivation, the bacterial cells were washed twice with PBS and resuspended in the same buffer. Inactivation effectiveness was verified by plating bacteria on MRS agar and incubation for 48 h at 37°C under anaerobic conditions. Lack of colony growth indicated complete inactivation. Three technical replicates were made (each in a triplicate). Deglycosylated bacterial cells were used in the adhesion assay to examine the influence of deglycosylation on the efficiency of adhesion to epithelial cells.

Inhibition of glycotransferases. *L. casei* Fyos was grown in the presence or absence (controls) of five different glycotransferase inhibitors: castanospermine, deoxymannojirimycin, kifunensine, swainsonine and deoxynojirimycin hydrochloride (1 mg/ml of MRS broth) for 18–20 h, 37°C under anaerobic conditions. Methyl-[^{3}H]-thymidine (5 µl/ml of broth, 60–90 Ci/mmol, 1 mCi/ml) was added to label the bacteria. After this time, bacteria were inactivated in 4% formaldehyde in PBS for 24 h at room temperature, washed two times with PBS and resuspended in HBSS. Three technical replicates were made (each in a triplicate). Inactivated bacterial cells were used in the adhesion assay to examine the influence of glycotransferase inhibition on the efficiency of adhesion to epithelial cells.

Cell surface proteome analysis. Proteomic analyses were performed using mass spectrometry. *L. casei* Fyos was grown in the presence of swainsonine (1 mg/1 ml

Table I
Composition of the Protein Deglycosylation Mix.

Enzyme	Hydrolyzed bounds	Number of enzymatic units added per 100 µl of reaction
O-Glycosidase	Catalyzes the removal of core 1 and core 3 *O*-linked disaccharides from glycoproteins.	400000 U
PNGase F	Cleaves between the innermost GlcNAc and asparagine residues of high mannose, hybrid, and complex oligosaccharides from *N*-linked glycoproteins unless α(1-3) core fucosylated	5000 U
Neuraminidase	Catalyzes the hydrolysis of terminal, non-reducing α2,3, α2,6, and α2,8 linked *N*-acetylneuraminic acid residues from glycoproteins and oligosaccharides.	500 U
β1-4 Galactosidase	Catalyzes the hydrolysis of terminal, non-reducing β1-4 linked D-galactopyranosyl residues from oligosaccharides and glycoproteins.	80 U
β-*N*-Acetylglucosaminidase	Catalyzes the hydrolysis of terminal, non-reducing β-*N*-Acetylglucosamine residues from oligosaccharides and glycoproteins.	40 U

of MRS broth) or without swainsonine (control). After incubation for 18–20 h, 37°C under anaerobic conditions, the cell wall proteome was isolated. The bacterial biomass derived from 10 ml of overnight culture was used for isolation. Bacteria were washed with PBS and resuspended in the buffer containing 50 mM Tris pH 7.5, 1 mM EDTA, 0.1% SDS and protease inhibitors (4-(2-aminoethyl) benzenesulfonyl fluoride, 2 mM/100 ml, aprotinin, 0.3 μM/100 ml, bestatine 130 μM/100 ml, E-64 14 μM/100 ml, leupeptin 1 μM/100 ml) to extract the external proteins. After 20 min of extraction, samples were centrifuged; proteins were extracted from the supernatant by adding trichloroacetic acid to the final concentration of 1% and sodium deoxycholate to the final concentration of 0.1%. Samples were incubated overnight at 4°C, then proteins were centrifuged at 20000 × g, washed in cold acetone, dried and solubilized in the loading buffer for Laemlii SDS-PAGE. Three biological replications of the experiment were made. The protein samples were stored frozen (–80°C). Protein samples were briefly resolved in SDS-PAGE and gel slabs with proteins were sent for analysis to the Laboratory of Mass Spectrometry, IBB PAS (Warsaw, Poland), where the samples were analyzed using tandem mass spectrometry.

Lactobacilli to Caco-2 cells adhesion assay. The adhesion assay was performed using Caco-2 cell line (according to Schmidt *et al.* 2010). Bacteria were labeled with methyl-[3H]-thymidine (60–90 Ci/mmol, 1 mCi/ml; Perkin Elmer, USA) at a volume of 5 μl/ml of broth and cultured for 18–20 h. After this time, the bacteria were washed twice with sterile HBSS and resuspended in the same buffer. The monolayers of differentiated Caco-2 cells were cultivated for three weeks on the PTFE filter (0.3 μm pore size) inserts in six-well tissue-culture dishes (Merck-Millipore, Poland) after inoculation of 2×10^6 viable cells (passage no. 49–52) per insert in 2.0 ml culture medium. The Caco-2 cell monolayers were washed once with 1 ml sterile HBSS before the adhesion assay. Bacteria were added to each well (5×10^8 cfu/ml of bacteria in 2.0 ml HBSS) and incubated at 37°C in an atmosphere of 10% CO2 for 60 min. After this time the monolayers were washed three times with sterile HBSS to remove the bacterial cells that did not adhere to Caco-2 cells. The radiolabeled bacteria in the density initially added for adhesion and the Caco-2 monolayer with adhered radiolabeled bacteria were lysed in 0.9 ml of 1% SDS. To complete lysis, 0.1 ml of 1 M NaOH was added and the lysate was incubated overnight at 60°C. The radioactivity of the lysed suspension was measured by liquid scintillation in Beckmann LS6500 after addition of the Hionic-Fluor scintillation cocktail (Perkin-Elmer, Poland). The bacterial adhesion efficiency was calculated based on the number of bacterial cells present after the adhesion test, relative to the number applied initially to the membrane with Caco-2 cells. Each assay was performed in triplicate.

Co-culture adhesion assay. Adhesion of lactic acid bacteria and *B. luti* DSM 14534 or *F. prausnitzii* DSM 17677 co-cultures was tested after incubation in the modified Anaerobe Basal Broth without a carbon (therefore, the only sources of carbon for *B. luti* DSM 14534 or *F. prausnitzii* DSM 17677 were exopolysaccharides produced by lactic acid bacteria). Initial density of each bacterial strain was 10^8 cfu/ml, the same volumes of 24 h-culture of lactic acid bacteria in the MRS broth and *B. luti* DSM 14534 or *F. prausnitzii* DSM 17677 72 h-culture in the Anaerobe Basal Broth were mixed, then washed two times with PBS and cultured for 18 h at 37°C (anaerobic jar) in the Anaerobe Basal Broth without carbon sources. After that time, the density of co-culture was recorded, using spectrophotometric measurement (wavelength of 600 nm). To prepare controls, the appropriate numbers of lactic acid bacteria and *B. luti* DSM 14534 or *F. prausnitzii* DSM 17677 were incubated separately under the same conditions and mixed immediately before the adhesion assay. The number of bacterial cells in culture was approximated by comparison of spectrophotometric measurements of culture density (600 nm wavelength) and inoculations of decimal decays of bacterial suspension. Measurements and cultures were performed in triplicates (18 h of lactobacilli cultures and 48 h of *F. prausnitzii* DSM 17677 and *B. luti* DSM 14534 cultures). Three biological replications were made. Co-cultures were used in the adhesion assay. The number of adhered bacterial cells was estimated by plating decimal dilutions of lysed Caco-2 cells. Lysis of Caco-2 cells was carried out by adding 1 ml of 0.1% SDS. Then, a Viscolase nuclease (A&A Biotechnology, Gdańsk, Poland) was added to the probes (1 U/ml) to degrade DNA released from lysed Caco-2 cells. Serially diluted samples were plated on MRS agar and incubated for 48 h, at 37°C under microaerophilic conditions (to avoid growth of *B. luti* DMS 14534 or *F. prausnitzii* DMS 17677, which are strictly anaerobic strains). After that time, colonies of lactic acid bacteria were counted and the effectiveness of adhesion was calculated. Each observed colony corresponded to one bacterial cell, which adhered to Caco-2 cells. The bacterial adhesion efficiency was calculated based on the number of bacterial cells present after the adhesion test, relative to the number of bacteria incubated with Caco-2 cells. The result was the percentage of bacteria that adhered to Caco-2 cells.

Statistical analysis. The t-test for dependent groups was used to compare the results obtained for experiment evaluating the effect of deglycosylation on lactobacilli adhesion to Caco-2. One-way analysis of variance (ANOVA) was used to analyze the data on

the influence of glycotransferases inhibition on adhesion efficiency to Caco-2 and the co-culture adhesion to Caco-2. The level of statistical significance was $p < 0.05$. The statistical calculations were performed using Statistica software.

Results

***L. rhamnosus* KL 53A and *L. casei* Fyos produced acidic heteroexopolysaccharides.** The exopolysaccharides of lactic acid bacteria were isolated from cells grown on MRS agar plates under anaerobic conditions at 37°C. The HPLC analysis revealed that both *L. rhamnosus* KL 53A and *L. casei* Fyos produced acidic heteropolysaccharides that differ in their monosaccharide composition. *L. rhamnosus* KL 53A produced EPS composed mainly of arabinose, glucose and galactose at a ratio of approximately 1:2:4 with a minor addition of maltose. *L. casei* Fyos produced EPS composed mainly of glucose, galactose and arabinose at a ratio of about 1:2:4 with a minor addition of ribose. This indicated that both strains share major constituents of EPS moieties (Table II).

Table II
Monosaccharides composition of the EPS produced by *L. rhamnosus* KL 53A and *L. casei* Fyos when grown on MRS agar (anaerobic conditions, 37°C, 18 h).

Monomer	% of a monomer in EPS isolated from the strain tested	
	Lactobacillus rhamnosus KL 53A	*Lactobacillus casei* Fyos
Maltose	0.71	0
Glucose	30.9	15.7
Galactose	53.7	31.4
Arabinose	14.6	51.6
Ribose	0	1.4

Deglycosylation of bacterial cell surface with a mixture of glycolytic enzymes reduced adhesion efficiency of *L. rhamnosus* KL 53A, but not *L. casei* Fyos. Two strains of lactic acid bacteria: *L. rhamnosus* KL 53A and *L. casei* Fyos, were used in this experiment. The metabolically labelled cells were fixed with formaldehyde and subsequently treated with the deglycosylation enzyme cocktail. Deglycosylated cells were incubated with the Caco-2 cell monolayer to estimate adhesion efficiency of the treated vs. untreated cells. A significant decrease ($p < 0.05$) in adhesion efficiency was observed only in the case of *L. rhamnosus* KL 53A (Fig. 1).

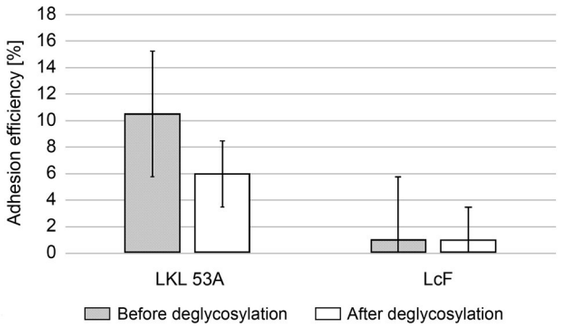

Fig. 1. Adhesion efficiency of the fixed bacteria before degycosylation and after deglycosylation. LKL 53A, *L. rhamnosus* KL 53A, LcF, *L. casei* Fyos. The bars represent average percentages of bacterial cells that adhered to differentiated Caco-2 enterocytes, the whiskers represent average adhesion ± standard error.

Inhibition of alpha-mannosidase with swainsonine increased adhesion efficiency of *L. casei* Fyos. We tested the influence of several glycotransferase inhibitors on adhesion efficiency of *L. casei* Fyos. Among the inhibitors tested (castanospermine, deoxymannojirimycin hydrochloride, swainsonine, kifunensine, and deoxynojirimycin hydrochloride) the treatement with swainsonine, an alpha-mannosidase inhibitor, resulted in a 2.8-fold increase of adhesion efficiency of the tested strain (Fig. 2). Although mannose was not found as a constituent of *L. casei* Fyos exopolysaccharide, the alpha-mannosidase encoding gene is commonly present in the genome of the species (Broadbent et al. 2012).

The cell surface proteome was not changed significantly by swainsonine treatment. To determine changes of the cell surface proteome after growth in the presence of swainsonine we performed the proteomic analysis by mass spectrometry. A comparison of relative numbers of identified proteins (n = 275) revealed a high correlation between control cells and those grown in the presence of swainsonine ($r = 0.957$, Fig. 3). The *L. casei* Fyos cells grown in the presence of the glycotransferase inhibitor showed a small (up to 2.25-fold) increase of several proteins: protein translocase subunit SecA, citrate lyase alpha-chain/citrate lyase, glycerol phosphate lipoteichoic acid synthase, glutamyl-tRNA(Gln) amidotransferase subunit A, the ATP synthase gamma chain, the ATP-dependent Clp protease proteolytic subunit/endopeptidase Clp, nitroreductase, 30S ribosomal protein S2, GMP synthase [glutamine-hydrolyzing]/glutamine amidotransferase, and the ATP synthase F1 sector beta subunit. This result suggests that the observed increase in adhesion efficiency after treatment with swainsonine was not a result of changes in the proteome as analyzed by mass spectrometry.

***F. prausnitzii* DSM 17677 affected adhesion of lactobacilli to enterocytes.** We tested the influence of coincubation of *Blautia luti* DSM 14534 and *F. prausnitzii* DSM 17677 with two strains of lactic acid bacteria, *L. casei* Fyos and *L. rhamnosus* KL 53A. A significant

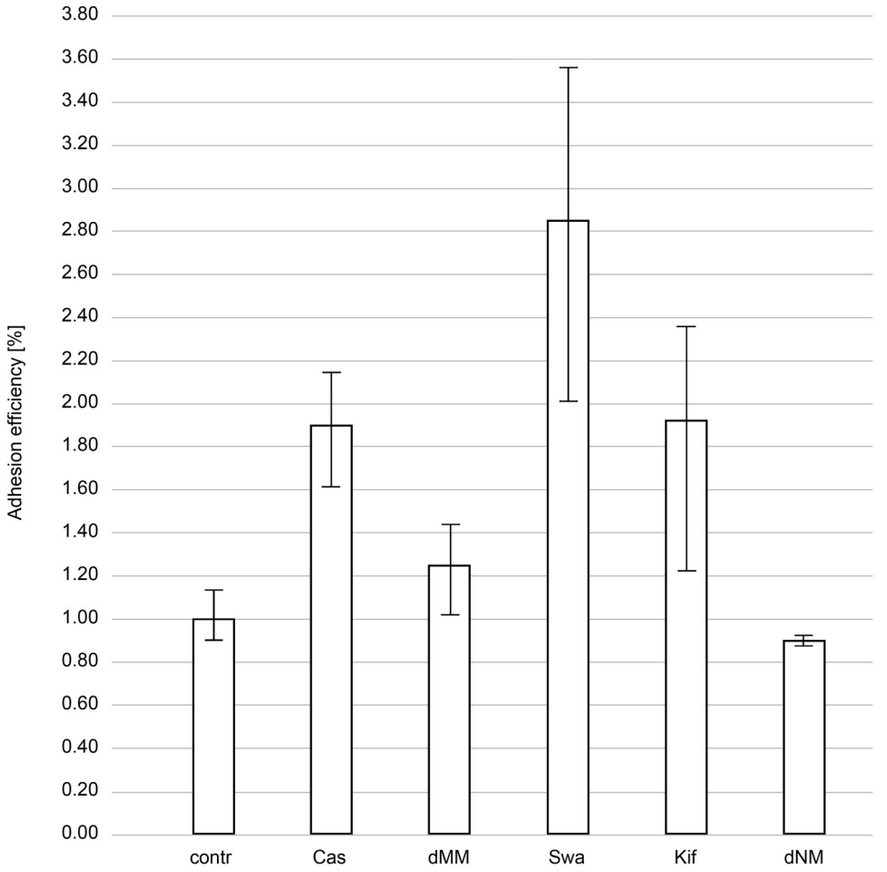

Fig. 2. Normalized to 100% adhesion efficiency of *L. casei* Fyos grown in the presence of glycosidase inhibitors: castanospermine (Cas), deoxymannojirimycin hydrochloride (dMM), swainsonine (Swa), kifunensine (Kif), deoxynojirimycin hydrochloride (dNM) and without inhibitor (contr). The bars represent average percentages of bacterial cells that adhered to differentiated Caco-2 enterocytes, the whiskers represent average adhesion ± standard error. Statistically significant changes ($p < 0.05$) are marked with asterisks (*).

increase ($p < 0.05$) in adhesion efficiency of lactobacilli was observed after incubation with *F. prausnitzii* DSM 17677 (1.3-fold increase for *L. casei* Fyos and 1.6-fold increase for *L. rhamnosus* KL 53A). *Blautia luti* caused no effect on adhesion ability of both lactobacillus strains (Fig. 4).

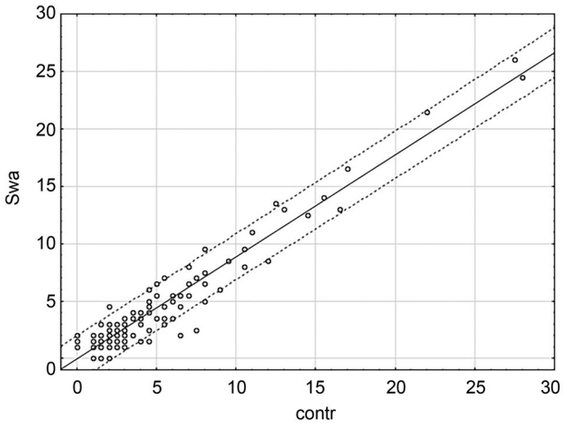

Fig. 3. A comparison of relative protein number in the *L. casei* Fyos proteomic mass spectrometry data of the control cells (contr) and of the cells grown in the presence of swainsonine (Swa).

Discussion

Many strains of intestinal microbiota exhibit the ability to degrade polysaccharides. The mechanisms they use may differ, but they always include the cell surface processing of a large glycan that precede the cellular import. *Firmicutes* produce polypeptides that bind carbohydrates and show catalytic functions. These proteins can bind EPS and release oligosaccharides, which are captured by cell wall proteins that import them into the bacterial cell (Cockburn and Koropatkin 2016). Both strains used in this study, *F. prausnitzii* DSM 17677 and *B. luti* DSM 14534, which metabolize polysaccharides (Lopez-Siles et al. 2012), belong to *Firmicutes,* but only *F. prausnitzii* DSM 17677 affected the adhesion of lactobacilli to enterocytes by enhancing this property.

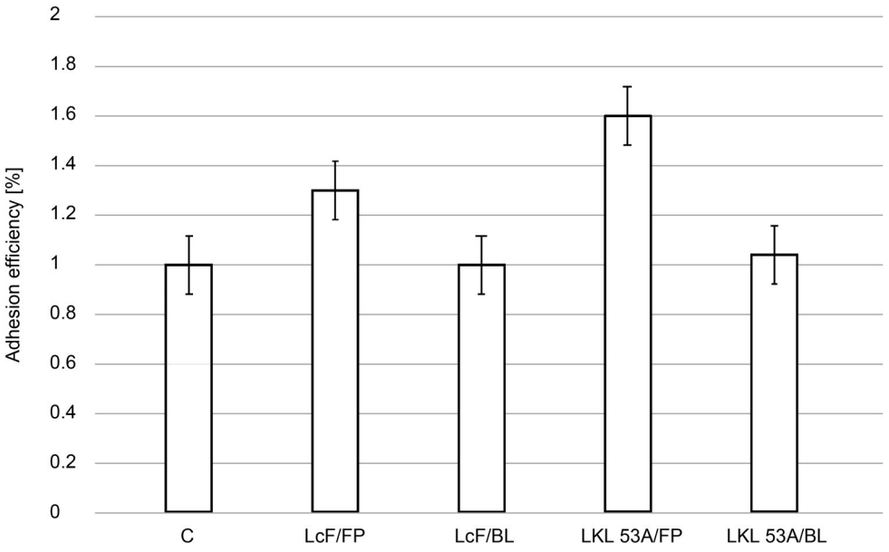

Fig. 4. Changes in adhesion efficiency to Caco-2 cells of two lactic acid bacteria strains co-cultured with anaerobic gut bacteria. Adhesion efficiency was normalized to 100%. Statistically significant changes ($p < 0.05$) are marked with asterisks (*). C – control: either *L. casei* Fyos or *L. rhamnosus* KL 53A; LcF/FP, *L. casei* Fyos co-cultured with *F. prausnitzii* DSM 17677; LcF/BL, *L. casei* Fyos co-cultured with *B. luti* DSM 14534; LKL 53A/FP, *L. rhamnosus* KL 53A co-cultured with *F. prausnitzii* DSM 17677; LKL 53A/BL, *L. rhamnosus* KL 53A co-cultured with *B. luti* DSM 14534.

In this study we demonstrated for the first time that *F. prausnitzii* DSM 17677 is able to affect adhesion of *L. rhamnosus* KL 53A and *L. casei* Fyos to enterocytes. *F. prausnitzii* is a phylogenetic group commonly found in human intestinal microbiome, accounting for approximately 5% of intestinal bacteria (Miquel et al. 2013). Two strains of probiotic lactobacilli used in this study synthesize acidic heteropolysaccharides, which remain attached to cell wall and most likely have an impact on adhesive ability of these bacteria. It was demonstrated by the use of deglycosylation enzymes (Fig. 1), glycosylase inhibitor treatment, and incubation with *F. prausnitzii* DSM 17677. This data shows a novel type of microbial interaction that can occur in the gastrointestinal tract and influence probiotic activity. Our finding is especially interesting in respect to the latest research of Desai et al. (2016) who demonstated that a dietary fiber-deprived gut microbiota degrades the colonic mucus barrier and enhances pathogen susceptibility.

In order to carry out experiments that required the inhibition of EPS synthesis and deglycosylation of lactobacilli, it was necessary to inactivate the bacterial cells synthesis during and after the experiments. The effect of inactivation on the adhesion of lactobacilli to Caco-2 cells has been thoroughly tested in our previous work (Markowicz et al. 2016). Formaldehyde inactivation was chosen due to the relatively low impact on the bacterial cell surface properties. It neither cause complete loss of protein activity nor reduce the adhesive properties, which was important for further analysis.

Incubation of bacteria with a mixture of deglycosylating enzymes resulted in a decreased efficiency of adhesion in *L. rhamnosus* KL 53A, which produces EPS composed mainly of arabinose, glucose and galactose with a small addition of maltose. It is likely that in this case EPS can act as an adhesion-promoting factor and when it was degraded (or partially degraded), adhesion of *L. rhamnosus* KL 53A was affected. There was no effect of enzymatic deglycosylation on *L. casei* Fyos adhesion; however, inclusion of the glycosylase inhibitor swainsonine resulted in an increased adhesion efficiency of the strain. Inhibition of EPS by synthesis could result in the exposure of surface adhesive proteins and, consequently, increased adhesion. In the case of *L. casei* Fyos, EPS can be an adhesion-reducing agent.

The deglycosylation of *L. casei* Fyos, using swainsonine, an α-(1→3)-mannosidase and α-(1→6)-mannosidase inhibitor (Table III), resulted in an increase of adhesion to enterocytes. No changes were observed in the bacterial proteome under the influence of this inhibitor. Swainsonine changed the EPS properties on the cell surface in *L. casei* Fyos but the cell surface proteome remained unchanged. However, we were unable to determine the difference in the EPS composition in the swainosine-treated and untreated cells; thus, we hypothesize that the increase of adhesion was due to the changes in EPS rather than the proteome of *L. casei* Fyos. Swainsonine affected the EPS of bacteria despite the lack of mannose in their composition, which may suggest that the α-(1→3)-mannosidase and α-(1→6)-mannosidase participate at some steps of EPS

Table III
Function of glycosidase inhibitors used to inhibit EPS synthesis in *L. casei* Fyos.

GT inhibitor	The enzyme inhibited
Castanospermine	β-glucosidase β-xylosidase α-mannosidase β-mannosidase Maltase-glucoamylase
Deoxymannojirimycin	α-(1→2)-mannosidase
Kifunensine	
Swainsonine	α-(1→3)-mannosidase α-(1→6)-mannosidase
Deoxynojirimycin hydrochloride	α-glucosidase Maltase-glucoamylase

synthesis, whereas mannose residues are not finally exposed outside of the cell. Mannose can, for example, be a precursor for EPS synthesis or be included in repeating units assembled in the cytoplasm, but not a part of the final EPS.

To the best of our knowledge this is the first report on a new type of the bacterial interaction i.e. properties of one bacterial strain influence the activity of the other strain towards their host. Enzymatic deglycosylation, EPS synthesis inhibition and incubation with *F. prausnitzii* DSM 17677 caused a different effect on adhesion of *L. rhamnosus* KL 53A and *L. casei* Fyos. The experiments have shown that both the EPS composition and their presence or absence may affect the adhesion of the LAB strains to enterocytes. Also, polysaccharides-metabolizing strains (e.g. *F. prausnitzii* DSM 17677) can affect the adhesion of lactobacilli (including probiotic strains) to the intestinal epithelium. EPS play an important role in the adhesion of beneficial lactobacilli to the intestinal epithelium; however, their synthesis pathways and functions remain poorly known when compared to those of pathogenic microorganisms. This bacterial interaction may explain differences in the host response to probiotic ingestion due to differences in the host microbiome composition.

Acknowledgments

This work was supported by the Polish National Science Centre grant No. N N312 272640 in years 2011–2014. We would like to thank Dr Ewa Sitkiewicz from Laboratory of Mass Spectrometry, IBB PAS (Warsaw, Poland) for assistance in proteome analysis.

Authors' Contributions

CK contributed to the adhesion assays, bacterial cell cultures, isolation and hydrolysis of EPS, isolation and preparation of proteins for (MS/MS) analysis, deglycosylation assay, glycotransferases inhibition assay data collection and interpretation, writing of the manuscript. MS contributed to HPLC analysis and MTS contributed to culture of the Caco-2 cells, conception of the idea, data collection and interpretation, drafting and writing of the manuscript. All read and approved the manuscript.

Literature

Broadbent JR, Neeno-Eckwall EC, Stahl B, Tandee K, Cai H, Morovic W, Horvath P, Heidenreich J, Perna NT, Barrangou R et al. 2012. Analysis of the *Lactobacillus casei* supragenome and its influence in species evolution and lifestyle adaptation. BMC Genomics. 13:533.

Cockburn DW, Koropatkin NM. 2016. Polysaccharide degradation by the intestinal microbiota and its influence on human health and disease. J Mol Biol. 428:3230–3252.

Comstock LE, and Kasper DL. 2006. Bacterial glycans: key mediators of diverse host immune responses. Cell. 126:847–850.

Desai MS, Seekatz AM, Koropatkin NM, Kamada N, Hickey CA, Wolter M, Pudlo NA, Kitamoto S, Terrapon N, Muller A et al. 2016. A dietary fiber-deprived gut microbiota degrades the colonic mucus barrier and enhances pathogen susceptibility. Cell 167: 1339–1353.

De Vuyst L, Degeest B. 1999. Heteropolysaccharides from lactic acid bacteria. FEMS Microbiol Rev. 23:153–177.

Furukawa N, Matsuoka A, Takahashi T, Yamanaka Y. 2000. Anti-metastic effect of kefir grain components on Lewis lung carcinoma and highly metastic B16 melanoma in mice. J Agric Sci. Tokyo Nogyo Daigaku 45:62–70.

Galle S, Arendt EK. 2014. Exopolysaccharides from sourdough lactic acid bacteria. Crit Rev Food. Sci. 54:891–901.

Gopal PK, Crow VL. 1993. Characterization of loosely associated material from the cell surface of *Lactococcus lactis* subsp. *cremoris* E8 and its phage-resistant variant strain 398. Appl Environ Microbiol. 59:3177–3182.

Korakli M, Ganzle MG, Vogel RF. 2002. Metabolism by bifidobacteria and lactic acid bacteria of polysaccharides from wheat and rye, and exopolysaccharides produced by *Lactobacillus sanfranciscensis*. J Appl Microbiol. 92:958–965.

Lebeer S, Verhoeven TLA, Francius G, Schoofs G, Lambrichts I, Dufrêne Y, Vanderleyden J, De Keersmaecker SCJ. 2009. Identification of a gene cluster for the biosynthesis of a long, galactose-rich exopolysaccharide in *Lactobacillus rhamnosus* GG and functional analysis of the priming glycosyltransferase. Appl Environ Microbiol. 75:3554–3563.

Lopez-Siles M, Khanb TM, Duncanc SH, Harmsenb HJM, Garcia--Gila LJ, Flintc HJ. 2012. Cultured representatives of two major phylogroups of human colonic *Faecalibacterium prausnitzii* can utilize pectin, uronic acids, and host-derived substrates for growth. Appl Environ Microbiol. 78:420–428.

Markowicz C, Kubiak P, Grajek W, Schmidt MT. 2016. Inactivation of *Lactobacillus rhamnosus* GG by fixation modifies its probiotic properties. Can J Microbiol. 62:72–82.

Miquel S, Martín R, Rossi O, Bermúdez-Humarán LG, Chatel JM, Sokol H, Thomas M, Wells JM, Langella P. 2013. *Faecalibacterium prausnitzii* and human intestinal health. Curr Opin Microbiol. 16: 255–261.

O'Toole PW, Cooney JC. 2008. Probiotic bacteria influence the composition and function of the intestinal microbiota. Interdiscip Perspect Infect Dis. 2008:175285.

Patel A, Prajapati JB. 2013. Food and health applications of exopolysaccharides produced by lactic acid bacteria. Adv Dairy Res 1:107.

Rios-Covian D, Cuesta I, Alvarez-Buylla JR, Ruas-Madiedo P, Gueimonde M, de los Reyes-Gavilán CG. 2016. *Bacteroides fragilis* metabolises exopolysaccharides produced by bifidobacteria. BMC Microbiology 16:150.

Ruas-Madiedo P, de los Reyes-Gavilán CG. 2005. Invited review: methods for the screening, isolation, and characterization of exopolysaccharides produced by lactic acid bacteria. J Dairy Sci. 88:843–856.

Ruas-Madiedo P, Gueimonde M, Margolles A, de los Reyes-Gavilan CG, Salminen S. 2006. Exopolysaccharides produced by probiotic strain modify the adhesion of probiotics and enteropathogens to human intestinal mucus. J Food Prot. 69:2011–2015.

Ryan PM, Ross RP, Fitzgerald GF, Caplice NM, Stanton C. 2015. Sugar-coated: exopolysaccharide producing lactic acid bacteria for food and human health applications. Food Funct. 6:679–693.

Schmid J, Sieber V, Rehm B. 2015. Bacterial exopolysaccharides: biosynthesis pathways and engineering strategies. Front Microbiol. 6:496.

Schmidt MT, Olejnik-Schmidt AK, Myszka K, Borkowska M, Grajek W. 2010. Evaluation of quantitative PCR measurement of bacterial colonization of epithelial cells. Pol J Microbiol. 59:89–93.

Stack HM, Kearney N, Stanton C, Fitzgerald GF, Ross RP. 2010. Association of beta-glucan endogenous production with increased stress tolerance of intestinal lactobacilli. Appl Environ Microbiol. 76:500–507.

Sun J, Le GW, Shi YH, Su GW. 2007. Factors involved in binding of *Lactobacillus plantarum* Lp6 to rat small intestinal mucus. Applied Microbiology. 44:79–85.

Touyama M, Jin JS, Kibe R, Hayashi H, Benno Y. 2015. Quantification of *Blautia wexlerae* and *Blautia luti* in human faeces by real-time PCR using specific primers. Beneficial Microbes. 6:583–590.

van Hijum SA, Kralj S, Ozimek LK, Dijkhuizen L, van Geel-Schutten IG. 2006. Structure-function relationships of glucansucrase and fructansucrase enzymes from lactic acid bacteria. Microbiol. Mol Biol. 70:157–176.

Welman AD, Maddox IS. 2003. Exopolysaccharides from lactic acid bacteria: perspectives and challenges. Trends Biotechnol. 21:269–274.

Wexler AG, Bao Y, Whitney JC, Bobay LM, Xavier JB, Schofield WB, Barry NA, Russell AB, Tran BQ, Goo YA et al. 2016. Human symbionts inject and neutralize antibacterial toxins to persist in the gut. Proc Natl Acad Sci USA. 113:3639–3644.

Polish Journal of Microbiology
2018, Vol. 67, No 3, 283–290
https://doi.org/10.21307/pjm-2018-033

ORIGINAL PAPER

Biohydrogen Production by Antarctic Psychrotolerant *Klebsiella* sp. ABZ11

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Submitted 2 November 2017, revised and accepted 20 February 2018

Abstract

Lower temperature biohydrogen production has always been attractive, due to the lower energy requirements. However, the slow metabolic rate of psychrotolerant biohydrogen-producing bacteria is a common problem that affects their biohydrogen yield. This study reports on the improved substrate synthesis and biohydrogen productivity by the psychrotolerant *Klebsiella* sp. strain ABZ11, isolated from Antarctic seawater sample. The isolate was screened for biohydrogen production at 30°C, under facultative anaerobic condition. The isolate is able to ferment glucose, fructose and sucrose with biohydrogen production rate and yield of 0.8 mol/l/h and 3.8 mol/g, respectively at 10 g/l glucose concentration. It also showed 74% carbohydrate uptake and 95% oxygen uptake ability, and a wide growth temperature range with optimum at 37°C. *Klebsiella* sp. ABZ11 has a short biohydrogen production lag phase, fast substrate uptake and is able to tolerate the presence of oxygen in the culture medium. Thus, the isolate has a potential to be used for lower temperature biohydrogen production process.

Key words: *Klebsiella* sp., biohydrogen, facultative psychrotolerant, oxygen uptake, carbohydrate consumption

Introduction

Hydrogen is an attractive alternative energy carrier due to the high energy density, and the cleaner by-products generated when used in automobiles (Khan et al. 2017). Biological hydrogen production is a technique of producing hydrogen through biological processes, using microorganisms as the biocatalysts. Among all the biological processes, bacterial dark fermentation is the most promising one, due to the high biohydrogen yield, and the ability to ferment different substrates to produce biohydrogen (Khan et al. 2017; Miandad et al. 2017).

Psychrophiles and psychrotolerant bacteria are abundant in the colder environment, e.g. Antarctica. Psychrophiles grow optimally at 20°C, and their fermentative processes have been considered beneficial due to the unique enzymes they possess (Corr and Murphy 2011). However, the reduced metabolic rate in psychrophiles is one key factor that affects substrate uptake and synthesis, which invariably affect the rate of substrate degradation and fermentative yield (Lettinga et al. 2001; Thauer et al. 2010; Lu et al. 2011). Psychrotolerant bacteria on the other hand, can grow above 20°C (Morita 1975; Pesciaroli et al. 2012). Thus, they are expected to be more useful for biohydrogen production at ambient temperature. Some psychrotolerant strains can thrive between 0–40°C (Pikuta et al. 2016), giving them an advantage over psychrophiles. Temperature

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contributes significantly to fermentative biohydrogen yield due to its influence on the rate of metabolism and enzyme activity (Hallenbeck et al. 2012). Antarctic seawater is subjected to a wide variation in environmental conditions which permits the survival of a wide range of bacteria, including the fermentative strains (Delille 1992). This study therefore focused on the isolation and characterization of psychrotolerant biohydrogen producing bacteria from Antarctic seawater.

More recently, a growing number of research has focused on biohydrogen production using cold-active bacteria isolated from Antarctica. For instance, Alvarez-Guzmán et al. (2016) investigated biohydrogen productivity of psychrophilic bacteria at 25°C under anaerobic condition. They reported a prolonged biohydrogen production lag phase and carbohydrate uptake after the start of fermentation. We hypothesized that utilizing facultative psychrotolerant bacteria with tolerance to mesophilic temperature may be a good option for improving substrate uptake and biohydrogen productivity of cold-adapted bacteria. Therefore, the objective of this study was to investigate the biohydrogen production ability of Antarctic facultative psychrotolerant bacteria under mesophilic temperature conditions. In addition, the influence of oxygen on the substrate uptake and biohydrogen productivity was also examined.

Experimental

Materials and Methods

Isolation of bacterial strains. Antarctic seawater was collected near Ryswyck Point, Anvers Island, Antarctic Peninsula (S64°39.189', W62°47.776') during the UTM Antarctic Research Expedition in February-March 2015. The bacteria was cultured on Marine Agar 2216 (Difco™, USA). The water samples (2 ml) was inoculated into 20 ml of the Marine Broth 2216 and incubated at 25°C for 7 days, before being transferred (0.1 µl) onto an agar plate using spread plate technique and incubated at 30°C for three days. Colonies obtained were then further sub-cultured on fresh agar plates to obtain pure isolates, before being preserved in 50% glycerol stock at –80°C for further use (Peeters et al. 2012).

Screening for biohydrogen production. The isolates were screened for biohydrogen production in 60 ml serum bottles containing 40 ml of Marine Broth 2216 as the fermentation medium. Each of the five isolates was used as an inoculum in separate serum bottles. The isolates were pre-cultured in the Marine Broth overnight, centrifuged and re-suspended in 0.1 M phosphate buffer pH 7.6. Then 4 ml of the culture was introduced into the fermentation media. Fermentation was carried out at 30°C for 24 h. Headspace gas (1 ml) was collected using airtight syringe (Agilent Technology) and analysed using a Gas Chromatography-Thermal Conductivity Detector (GC-TCD) (Agilent 7890B) equipped with five columns (two Hayesep Q80/100 SS, one Hayesep Q 80/200, and two Molsieve 5A 60/80 SS). All experiments were performed in triplicates. Among the five isolates screened for biohydrogen production, only one strain (ABZ11) which showed significant biohydrogen production was selected for further identification and characterization.

Growth profile. To investigate the growth profile of the isolate, it was first cultured in Marine Broth under agitation condition (100 × g) and incubated at 30°C for 30 h. Cells at optical density at 600 nm (OD_{600}) of 1.0 (10 ml) was introduced into 100 ml sterile Marine Broth in a conical flask and incubated at 30°C. The culture (1 ml) was then transferred into pre-weighed microcentrifuge tubes before being centrifuged at 10 000 × g for 5 min and dried at 40°C overnight. The average final dry cell weight was used to represent the growth of the isolate (Silvaa et al. 2016).

Biochemical tests and electron microscopy. ABZ11 was characterized using Gram staining, capsule staining, and blood haemolysis test. Blood haemolysis test was performed on 5% sheep blood agar and incubated at 37°C for 48 h (Shirron et al. 2008). The morphology of ABZ11 was also examined using scanning electron microscopy (SEM) (JEOL JSM-6390LV) with accelerating voltage of 10 kV (Xiong et al. 2015). Cells for SEM was prepared by centrifuging 5 ml of the overnight bacterial culture at 10 000 × g for 5 min. Cell pellet was washed three times with 1 ml of 0.1 M phosphate buffer pH 7.6. The cells were fixed for 2 h with 2.5% glutaraldehyde and dried in a critical point dryer (Leica EM CPD300).

Identification using 16S rRNA analysis. The genomic DNA of ABZ11 was extracted using Wizard® Genomic DNA Purification Kit (Promega) according to manufacturer's instruction. The 16S rRNA gene was then amplified in a thermocycler (Eppendorf AG22331) using forward primer 27-F: 5'-AGAGTTTGATCCTGGCTCAG-3' and a reverse primer 1492R: 5'-CGGTTACCTTGTTACGACTT-3'. PCR was set at the following condition: 94°C for 4 min, 30 cycles at 94°C for 1 min, 48°C for 30 sec, and 68°C for 2 min, and kept at 4°C for amplification. The PCR products obtained were purified and sequenced by First Base Laboratories (Malaysia) Sdn. Bhd. The partial 16S rRNA gene sequence obtained was analysed using the BLAST tool at NCBI database, by comparing with the sequences in the Genbank database. Phylogenetic tree was constructed using the neighbor-joining method (Saitou and Nei 1987). Specific taxa of the organism was collapsed in less than 50% replicates from 1050 bootstrap replicates (Felsenstein 1985). The analysis was conducted using MEGA7 (Kumar et al. 2016).

Temperature range for optimum growth. Overnight culture of ABZ11 with OD_{600} of 1.0 was serially diluted to 10^{-5}, before 0.1 ml was plated by spread plate method and incubated at different temperatures (20, 30, 37, 40 and 45°C). All experiments were prepared in triplicates and colonies between 30 and 300 were considered for determination of cell viable numbers in CFU/ml (Pesciaroli et al. 2012).

Oxygen uptake capability. Oxygen uptake experiments were performed in a reconstituted medium containing 5 g/l glucose and 0.2 g/l beef extract in 0.1 M phosphate buffer pH 7.6, with trace elements (in g/l) $ZnCl_2$; 0.07, $MnCl_2 \cdot 4H_2O$; 0.1, H_3BO_3; 0.06, $CoCl_2 \cdot 6H_2O$; 0.2, $CuCl_2 \cdot 2H_2O$; 0.02, $NiCl_2 \cdot 6H_2O$; 0.025 and $NaMoO_4 \cdot 2H_2O$; 0.035 (Liu and Fang 2007). The medium (40 ml) was dispensed into 60 ml universal bottles before being inoculated with 4 ml of the overnight culture of the ABZ11. Afterwards, the bottles were tightly covered and sealed with parafilm and incubated at 25°C for 24 h. Dissolved oxygen uptake by the isolate were measured at 2, 4, 6, and 24 hours. Dissolved oxygen level was measured with a DO meter (Mettler Toledo FG4) (Santana 2008). All experiments were performed in triplicate and fermentation was carried out under static condition to avoid introducing oxygen into the medium.

Biohydrogen production using different carbon sources. Different carbon sources were tested, namely glucose, sucrose and fructose at 5, 7.5, 10 and 12.5 g/l concentrations. Beef extract and trace element solution were added as nitrogen and mineral source, as described above. The C/N ratio was fixed at 1/30. Fermentation was initiated by adding 10% (v/v) of the fresh culture (1.0 OD_{600}) of ABZ11 into 40 ml of the sterile medium in 60 ml serum bottles sealed with rubber stopper and aluminium cap (Islam et al. 2006). Experiments were prepared in triplicate and incubation was carried out at 30°C for 48 h. Headspace gas (1 ml) was collected with an airtight syringe and analysed in a GC-TCD as described above at different time intervals. Carbohydrate consumption was determined by 3,5-dinitrosalicylic acid (DNS) assay (Miller 1959). Growth profile was investigated as described above.

Kinetic analysis. Biohydrogen productivity and cell growth during the fermentation were evaluated using two equations $Y_{P/S}$ and $Y_{X/S}$ to determine whether the substrate taken up was channelled towards growth or product formation (Hahn-Hägerdal 1994; Silvaa et al. 2016). In the equations, biohydrogen yield ($Y_{P/S}$) refers to the fraction of the biohydrogen produced in (mol/l) and the substrate consumed (mg/ml).

$$Y_{P/S} = \frac{P_{max} - P_o}{S_{max} - SP_{max}}$$

Where Y = yield, P = product and S = substrate consumed (glucose, fructose and sucrose). P_{max} = biohydrogen produced at 48 hour, S_{max} = maximum carbohydrate concentration, P_o = biohydrogen produced at initial fermentation and SP_{max} = carbohydrate consumed at maximum biohydrogen production period.

While growth yield ($Y_{X/S}$) was defined as the fraction of the dry cell weight (g/ml) and the substrate consumed (mg/ml).

$$Y_{X/S} = \frac{X_{max} - X_o}{S_{max} - SX_{max}}$$

Where Y = yield, X = dry cell weight and S = carbohydrate consumed. X_{max} = maximum growth rate, X_o = initial dry cell weight, S_{max} = carbohydrate consumed at maximum growth period and SX_o = carbohydrate concentration at initial growth period.

Statistical analysis. Statistical analysis (analysis of variance, ANOVA) was carried out using SPSS software, version 24. ANOVA tests were applied at the significance level of $p < 0.05$. The data were from three independent experiments.

Results

Isolation and characterization of biohydrogen psychrotolerant bacteria. Five pure colonies were obtained from the Antarctic seawater sample, with milky mucoid color. All isolates grew on Marine Agar plates after three days incubation. These isolates were designated ABZ4, ABZ7, ABZ10, ABZ11 and ABZ12.

Out of the five isolates screened for biohydrogen production, only two isolates (Fig. 1) is able to produce biohydrogen, namely ABZ11 (0.49 mol H_2/mol glucose) and ABZ4 (0.02 mol H_2/mol glucose). Due to higher biohydrogen production, ABZ11 was selected for further characterizations.

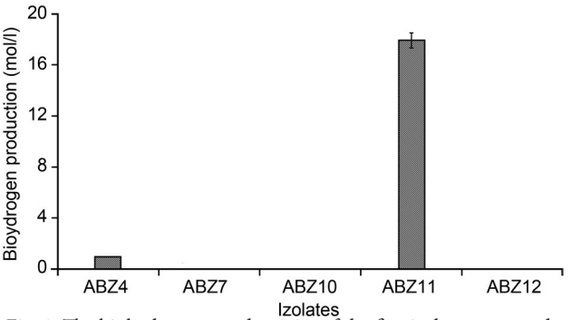

Fig. 1. The biohydrogen productivity of the five isolates screened.

ABZ11 has a short growth lag phase (1 h) (Fig. 2) but a longer stationary phase between 2 and 18 h of incubation. The cells of ABZ11 are rod-shaped, with an approximate length of 1.7 µm and diameter of 0.4 µm (Fig. 3). Capsule staining revealed no capsule around the cells as a result of the absence of visible halo zones after staining (Fig. 4).

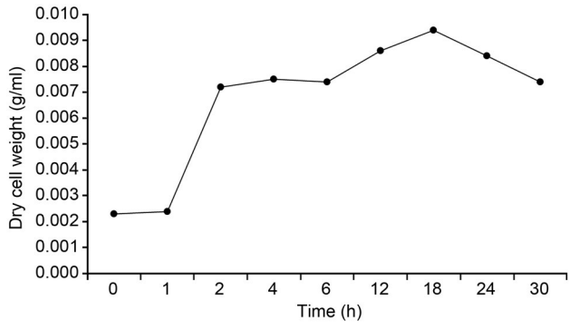

Fig. 2. Mean dry cell weight (DCW) of *Klebsiella* sp. ABZ11 during growth at 30°C.

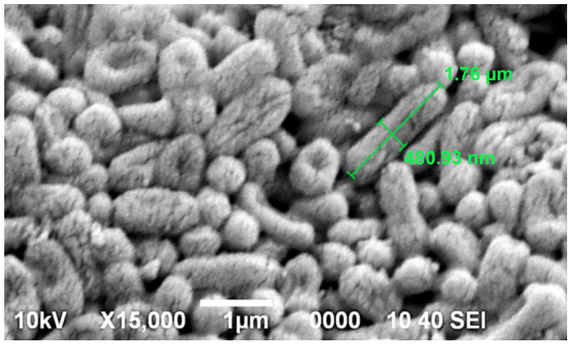

Fig. 3. Scanning electron micrograph of *Klebsiella* sp. ABZ11. Bar represents 1 µm scale.

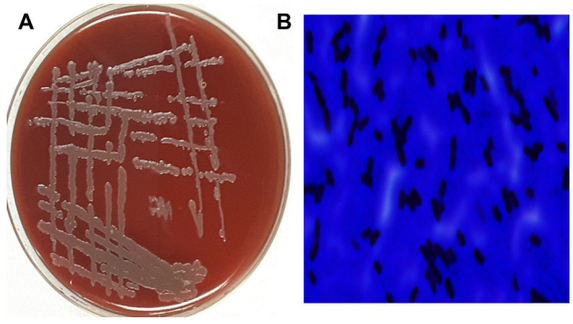

Fig. 4. A. Growth of *Klebsiella* sp. ABZ11 on blood agar plate. No blood haemolysis was observed after 48 h incubation at 30°C. B. Capsule staining of the ABZ11. No capsule is visible around the cells suggesting the non-pathogenicity of this strain.

Analysis of the partial 16S rRNA sequence showed that ABZ11 is closely related to *Klebsiella pneumoniae* (99% identity). The isolate was thus designated *Klebsiella* sp. ABZ11, with a Genbank accession number KX266892. Based on the phylogenetic relationship identified using neighbor-joining method, ABZ11 cluster with *Klebsiella pneumoniae* subsp. *rhinoscleromatis* R-70 strain on the same taxon among the 13 closely related strains that were used (Fig. 5).

Temperature tolerance of the *Klebsiella* sp. ABZ11 was tested by observing the viable cells after incubation at several different temperatures. ABZ11 is able to grow

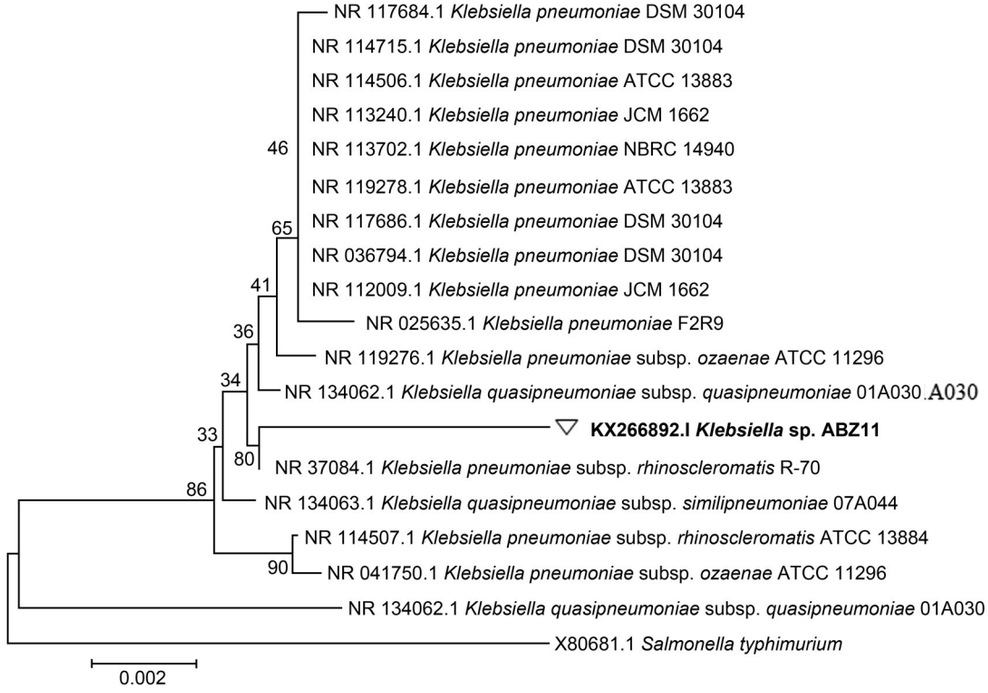

Fig. 5. Phylogenetic tree constructed using the partial 16S rRNA gene sequences by neighbor-joining method (19 nucleotides, 1303 data sets, branch length of 0.033 and all gaps removed) showing the relationship between ABZ11 and the 17 most closely related reference species. The gene sequence of *Salmonella typhimurium* X80681 was used as the outgroup. Bootstrap values (expressed as percentages of 1050 replications) are shown at the branch points. Bar represents 0.002 substitutions per nucleotide position.

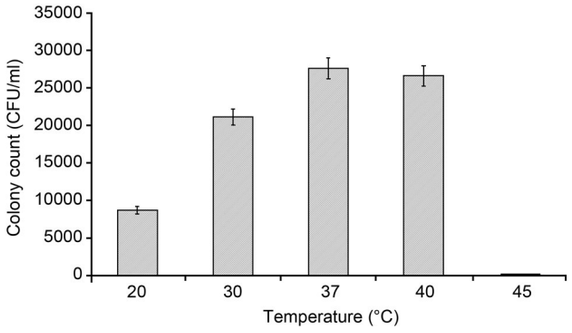

Fig. 6. Mean growth temperature range and optimum temperature of *Klebsiella* sp. ABZ11.

Biohydrogen production using different carbon sources. Biohydrogen production was observed to start after 2 hours of fermentation for all the substrate concentration except 12.5 g/l sucrose concentration that begins production at the 4th hour. Table I shows the summary of the biohydrogen production of bacterial with different concentrations of the carbohydrate sources. Meanwhile, the biohydrogen production was observed to increase with fermentation time for all the carbohydrate concentrations with maximum productivity observed at different time. Productivity was also observed to decrease with increased substrate concentration. A maximum biohydrogen of 38.55 mol/l

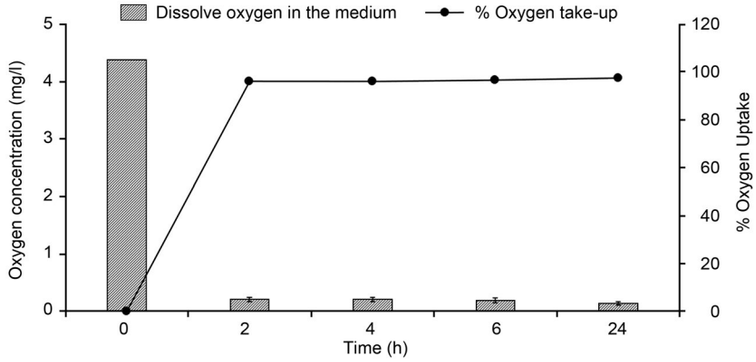

Fig. 7. Oxygen uptake by ABZ11 at different time intervals.

at 20, 30, 37, and 40°C, but no growth was observed at 45°C (Fig. 6). The optimum growth was observed at 37°C after three days of incubation.

Oxygen tolerance of ABZ11 was then investigated by measuring the level of dissolved oxygen in the culture medium. An oxygen uptake of 4.17 ± 0.03 mg/l was observed after 2 h, representing 95% of the oxygen in the medium that was scavenged by *Klebsiella* sp. ABZ11 (Fig. 7). Meanwhile, oxygen content was slightly reduced by 0.5% in 6 h and 0.02% in 24 h leading to a total of 96.8% of oxygen uptake in 24 h.

observed at 10 g/l was significantly ($p > 0.05$) different from the productivity of other substrate concentrations. Further, carbohydrate consumption started immediately with 74% of the total carbohydrate consumed after 2 h (Fig. 8). It was also observed that the carbohydrate in the medium was totally exhausted before 48 h in all tested parameters.

Biohydrogen yield in relation to the biomass during fermentation is 24.09 mol/mg while cell growth in relation to substrate utilization is 0.532 g/mg, at 10 g/l of glucose (Fig. 9). The maximum biohydrogen

Table I
Biohydrogen production and kinetic parameters of substrate utilization by *Klebsiella* sp. ABZ11.

	Carbohydrate concentrations (g/l)		5	7.5	10	12.5
Biohydrogen production at initial incubation time and at maximum production (mol/l)	Glucose	Initial	0.21 ± 0.00	0.21 ± 0.02	2.35 ± 0.03	0.77 ± 0.01
		Maximum	26.23 ± 2.18	23.80 ± 3.29*	38.55 ± 2.19*	21.11 ± 0.14*
	Fructose	Initial	0.21 ± 0.00	0.47 ± 0.05	0.65 ± 0.46	0.81 ± 0.09
		Maximum	25.47 ± 2.02	22.77 ± 2.01	19.97 ± 1.60	16.97 ± 0.12
	Sucrose	Initial	0.21 ± 0.00	0.23 ± 0.04	0.23 ± 0.01	4.52 ± 0.36
		Maximum	28.24 ± 2.96*	22.37 ± 2.19	18.21 ± 0.77	15.91 ± 1.49
Ketic analysis	$Y_{P/S}$ (mol/mg)		23.53	17.00	24.09	11.11
	$Y_{X/S}$ (g/mg)		0.170	0.424	0.532	0.205

* Maximum biohydrogen production in the group and the productivity considered for the kinetic

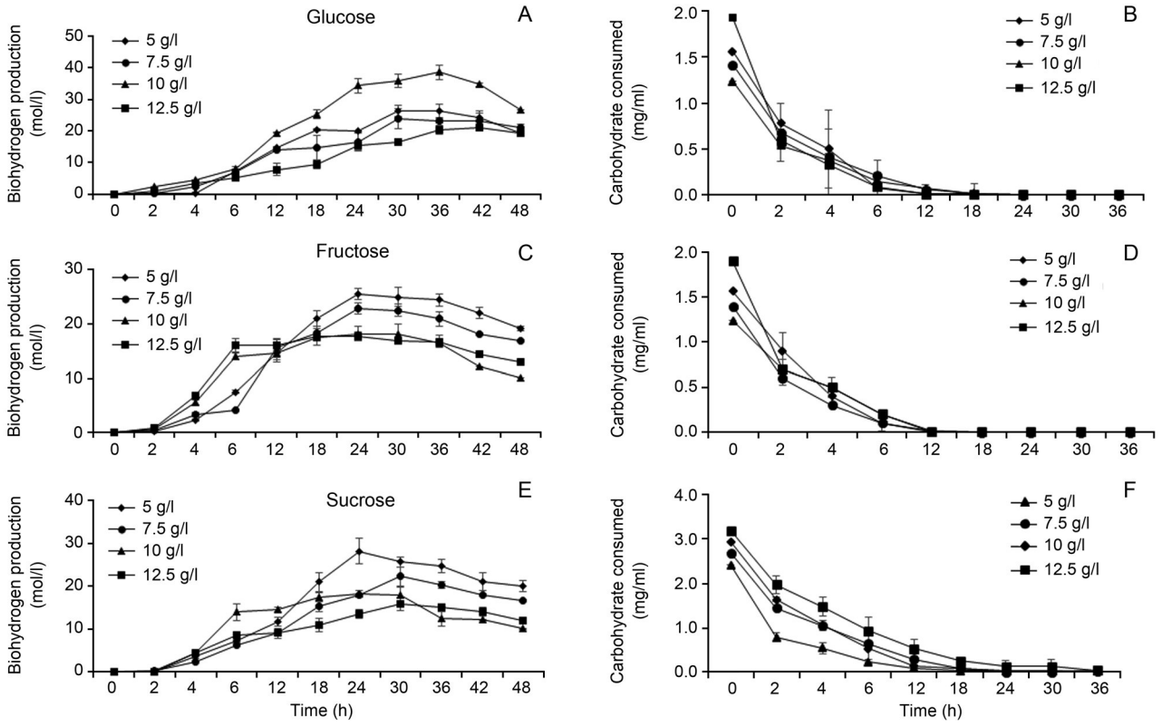

Fig. 8. Average biohydrogen productivity and carbohydrate uptake after 48 h fermentation of glucose (A, B), fructose (C, D) and sucrose (E, F) by *Klebsiella* sp. ABZ11.

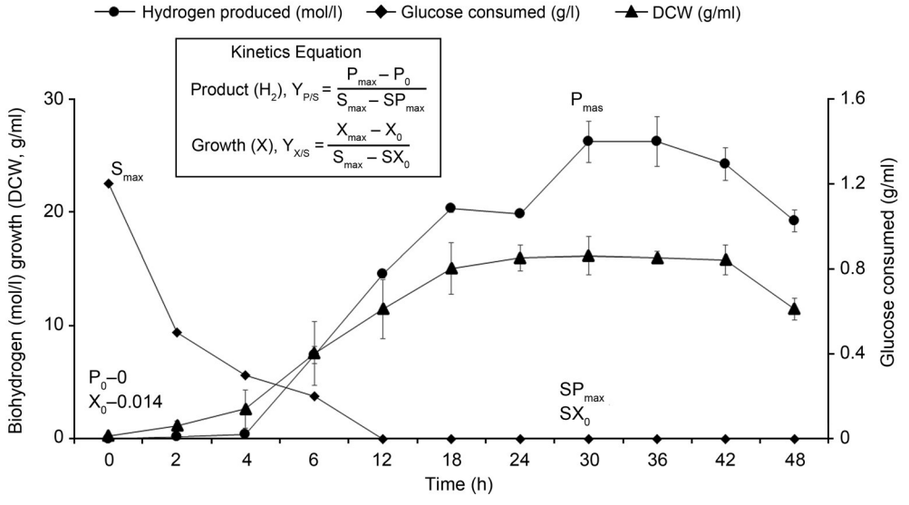

Fig. 9. Biohydrogen production kinetic of *Klebsiella* sp. ABZ11 at 5 g/l glucose concentration.

production and kinetic data for all the parameters investigated are summarized in Table I.

Discussion

Generally, there is a prolonged period between the fermentation start time and the beginning of biohydrogen production in psychrophilic bacteria, providing a challenge for application in lower temperature biohydrogen reactors. This study was thus initiated to overcome this challenge by using Antarctic psychrotolerant bacteria, with higher metabolic activity in order to improve its substrate uptake and biohydrogen production. We have successfully isolated a psychrotolerant, biohydrogen-producing *Klebsiella* sp. (closely related to *Klebsiella pneumoniae*) from Antarctic seawater. This *Klebsiella* sp. strain ABZ11 has the ability to withstand

a wide growth temperature, with optimum at 37°C, suggesting that this strain is psychrotolerant (Carrión et al. 2011; Lavin et al. 2016).

Klebsiella spp. has previously been isolated from Antarctic sea animals, samples obtained from islands, as well as other environments such as sewage sludge and hot springs (Minnan et al. 2005; Kargel et al. 2008; Brat et al. 2016). The diverse environmental conditions from which this genus was isolated suggest the unique physiological characteristics that allows their survival. *Klebsiella* sp. ABZ11 that we isolated from Antarctic seawater is devoid of capsules and does not show the ability to lyse blood cells, suggesting the non-pathogenic nature of this strain.

The carbon sources tested in the fermentation experiment were glucose, fructose and sucrose. The highest biohydrogen production of 38.55 ± 2.19 mol/l was observed at 10 g/l glucose concentration, at pH 5.6. A decrease in biohydrogen production with the increase in substrate concentration was also observed in almost all carbon sources tested, as reported previously (Ginkel et al. 2001; Kamalaskar et al. 2010) except for glucose. The optimum glucose concentration of 10 g/l observed in this study is in agreement with mesophilic *Bacillus* sp. FS2011 and *Clostridium beijerinckii* Fanp3 strains (Pan et al. 2008; Song et al. 2013) but differs with other work on hydrogen producing *Klebsiella* strains (Niu et al. 2010; Chookaew et al. 2012). Strain ABZ11 also shows high carbohydrate uptake, with 74% of glucose consumed after 2 h of fermentation due to high substrate conversion efficiency attributed to the incubation temperature and oxygen, before complete utilization around 48 h.

Biohydrogen production was detected within the exponential and stationary phase with maximum production found in the stationary phase of *Klebsiella* sp. ABZ11 growth, indicating that hydrogen production is more of a secondary metabolite in its fermentative process. This is similar to the report on biohydrogen production by mesophilic *Clostridium* sp. DMHC-10 (Kamalaskar et al. 2010). More importantly, strain ABZ11 shows a very short production lag phase, rapid carbohydrate uptake, and is able to substantially decrease culture oxygen level in a short period of time. Our findings thus suggest an improved metabolic biosynthesis for biohydrogen production compared to the psychrophiles and psychrotolerant strains (Alvarez-Guzmán et al. 2016). There is a possibility that *Klebsiella* sp. ABZ11 possesses oxygen tolerant hydrogenases, as previously reported in *K. oxytoca* HP1 (Minnan et al. 2005). The improvement in metabolic activity yielding early productivity may be attributed to the temperature condition at which the fermentation was performed. The rapid decrease in oxygen concentration in the fermentation medium could be another factor that influences the rate of the subsequent carbohydrate hydrolysis due to release of large extracellular hydrolytic enzymes and increase in their activity, which in turn increases hydrogen biosynthesis. A small amount of oxygen in the fermentation medium has been shown before to enhance the rate of substrate hydrolysis (Johansen and Bakke 2006; Ramos and Fdz-Polanco 2013).

Klebsiella sp. ABZ11 biohydrogen production kinetics show that the strain is inclined towards biohydrogen production rather than growth at the stationary phase, under the experimental conditions described here. Experiments performed under batch fermentation revealed the potential of this strain to be able to utilize different carbon sources for biohydrogen production. In the future, optimization of experimental parameters may further improve the biohydrogen-producing ability of this facultative psychrotolerant bacteria.

Conclusions

A psychrotolerant, facultative, oxygen-insensitive biohydrogen-producing bacterial strain has successfully been isolated from Antarctic seawater. The strain, identified as *Klebsiella* sp. ABZ11, has been investigated in terms of its biohydrogen productivity, temperature tolerance, and dissolved oxygen uptake. This strain shows up to 95% oxygen uptake, and can tolerate growth temperature of 20–40°C, with optimum growth observed at 37°C. Maximum biohydrogen productivity of 38.55 mol/l was observed at 30°C. This study demonstrates the feasibility of using a psychrotolerant bacterial strain for biohydrogen production at lower temperature, with high oxygen tolerance.

Acknowledgements

This work was supported by UTM Flagship Research Grant (grant numbers 01G27, 00G98, 02E13) and UTM Tier 1 Research University Grant (grant number 07H31). A.M. would like to acknowledge the Tertiary Education Trust Fund (Tetfund) Nigeria and IBB University, Lapai, Niger State, Nigeria for PhD scholarship provided.

Literature

Alvarado-Cuevas ZD, López-Hidalgo AM, Ordoñez LG, Oceguera-Contreras E, Ornelas-Salas JT, De León-Rodríguez A. 2015. Biohydrogen production using psychrophilic bacteria isolated from Antarctica. Int J Hydrog Energy. 40(24):7586–7592.

Alvarez-Guzmán CL, Oceguera-Contreras E, Ornelas-Salas JT, Balderas-Hernández VE, De León-Rodríguez A. 2016. Biohydrogen production by the psychrophilic G088 strain using single carbohydrates as substrate. Int J Hydrog Energy. 41(19):8092–8100.

Brat K, Sedlacek I, Sevcikova A, Merta Z, Laska K, Sevcik P. 2016. Imported anthropogenic bacteria may survive the Antarctic winter and introduce new genes into local bacterial communities. Pol Polar Res. 37(1):89–104.

Pan CM, Fan YT, Zhao P, Hou HW. 2008. Fermentative hydrogen production by the newly isolated *Clostridium beijerinckii* Fanp3. Int J Hydrog Energy. 33(20):5383–5391.
Carrión O, Miñana-Galbis D, Montes MJ, Mercadé E. 2011. *Pseudomonas deceptionensis* sp. nov., a psychrotolerant bacterium from the Antarctic. Int J Syst Evol Microbiol. 61(10):2401–2405.
Chookaew T, Sompong O, Prasertsan P. 2012. Fermentative production of hydrogen and soluble metabolites from crude glycerol of biodiesel plant by the newly isolated thermotolerant *Klebsiella pneumoniae* TR17. Int J Hydrog Energy. 37(18):13314–13322.
Corr MJ, Murphy JA. 2011. Evolution in the understanding of [Fe]-hydrogenase. Chem Soc Rev. 40(5):2279–2292.
Delille D. 1992. Marine bacterioplankton at the Weddell Sea ice edge, distribution of psychrophilic and psychrotrophic populations. In: Hempel G. (eds) Weddell Sea Ecology. Springer, Berlin, Heidelberg.
Felsenstein J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution. 39(4):783–791.
Ginkel SV, Sung S, Lay JJ. 2001. Biohydrogen production as a function of pH and substrate concentration. Env Sci Technol. 35(24):4726–4730.
Hahn-Hägerdal B, Jeppsson H, Skoog K, Prior BA. 1994. Biochemistry and physiology of xylose fermentation by yeasts. Enzyme Microb Technol. 16(11):933–943.
Hallenbeck PC, Abo-Hashesh M, Ghosh D. 2012. Strategies for improving biological hydrogen production. Bioresource Technol. 110:1–9.
Islam R, Cicek N, Sparling R, Levin D. 2006. Effect of substrate loading on hydrogen production during anaerobic fermentation by *Clostridium thermocellum* 27405. Applied Microbiol Biotechnol. 72:576–583.
Johansen JE, Bakke R. 2006. Enhancing hydrolysis with microaeration. Water Sci Technol. 53(8):43–50.
Kamalaskar LB, Dhakephalkar PK, Meher KK, Ranade DR. 2010. High biohydrogen yielding *Clostridium* sp. DMHC-10 isolated from sludge of distillery waste treatment plant. Int J Hydrog Energy. 35(19):10639–10644.
Kargel JS, Dimas VM, Kao DS, Heggers JP, Chang P, Phillips LG. 2008. Empiric antibiotic therapy for seawater injuries: A four-seasonal analysis. Plast Reconstr Surgery. 121(4):1249–1255.
Khan MA, Ngo HH, Guo W, Liu Y, Zhang X, Guo J, Chang SW, Nguyen DD, Wang J. 2017. Biohydrogen production from anaerobic digestion and its potential as renewable energy. Renew Energy. in press.
Kumar S, Stecher G, Tamura K. 2016. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. Mol Biol Evol. 33(7):1870–1874.
Lavin PL, Yong ST, Wong CM, De Stefano M. 2016. Isolation and characterization of Antarctic psychrotroph *Streptomyces* sp. strain INACH3013. Antarct Sci. 28(6):433–442.
Lettinga G, Rebac S, Zeeman G. 2001. Challenge of psychrophilic anaerobic wastewater treatment. Trends Biotechnol. 19(9):363–370.
Liu F, Fang B. 2007. Optimization of bio-hydrogen production from biodiesel wastes by *Klebsiella pneumoniae*. Biotechnol J. 2(3): 374–380.
Lu L, Ren N, Zhao X, Wang H, Wu D, Xing D. 2011. Hydrogen production, methanogen inhibition and microbial community structures in psychrophilic single-chamber microbial electrolysis cells. Energy Env Sci. 4(4):1329–1336.
Miandad R, Rehan M, Ouda O, Khan M, Shahzad K, Ismail I, Nizami A. 2017. Waste-to-hydrogen energy in Saudi Arabia: challenges and perspectives. Biohydrogen Production: Sustainability of Current Technology and Future Perspective. India: Springer. p. 237–252.
Miller GL. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Chem. 31(3):426–428.
Minnan L, Jinli H, Xiaobin W, Huijuan X, Jinzao C, Chuannan L, Fengzhang Z, Liangshu X. 2005. Isolation and characterization of a high H_2-producing strain *Klebsiella oxytoca* HP1 from a hot spring. Res Microbiol. 156(1):76–81.
Morita RY. 1975. Psychrophilic bacteria. Bacteriol Rev. 39(2): 144–167.
Niu K, Zhang X, Tan WS, Zhu ML. 2010. Characteristics of fermentative hydrogen production with *Klebsiella pneumoniae* ECU-15 isolated from anaerobic sewage sludge. Int J Hydrog Energy. 35(1):71–80.
Peeters K, Verleyen E, Hodgson DA, Convey P, Ertz D, Vyverman W, Willems A. 2012. Heterotrophic bacterial diversity in aquatic microbial mat communities from Antarctica. Polar Biol. 35(4):543–554.
Pesciaroli C, Cupini F, Selbmann L, Barghini P, Fenice M. 2012. Temperature preferences of bacteria isolated from seawater collected in Kandalaksha Bay, White Sea, Russia. Polar Biol. 35(3):435–445.
Pikuta EV, Menes RJ, Bruce AM, Lyu Z, Patel NB, Liu Y, Hoover RB, Busse HJ, Lawson PA, Whitman WB. 2016. *Raineyella antarctica* gen. nov., sp. nov , a psychrotolerant, d-amino-acid-utilizing anaerobe isolated from two geographic locations of the Southern Hemisphere. Int J Syst Evol Microbiol. 66(12):5529–5536.
Ramos I, Fdz-Polanco M. 2013. The potential of oxygen to improve the stability of anaerobic reactors during unbalanced conditions: results from a pilot-scale digester treating sewage sludge. Bioresour Technol. 140:80–85.
Saitou N, Nei M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol. 4(4):406–425.
Santana M. 2008. Presence and expression of terminal oxygen reductases in strictly anaerobic sulfate-reducing bacteria isolated from salt-marsh sediments. Anaerobe. 14(3):145–156.
Shirron N, Korem M, Shuster A, Leikin-Frenkel A, Rosenberg M. 2008. Effect of alcohol on bacterial hemolysis. Curr Microbiol. 57(4):318–325.
Silvaa AS, Júniora AMO, de Farias Silvab CE, Abud AKS. 2016. Inhibitors Influence on Ethanol Fermentation by *Pichia stipitis*. Chem Eng. 49:367–372.
Song ZX, Li WW, Li XH, Dai Y, Peng XX, Fan YT, Hou HW. 2013. Isolation and characterization of a new hydrogen-producing strain *Bacillus* sp. FS2011. Int J Hydrog Energy. 38(8):3206–3212.
Thauer RK, Kaster AK, Goenrich M, Schick M, Hiromoto T, Shima S. 2010. Hydrogenases from methanogenic archaea, nickel, a novel cofactor, and H_2 storage. Annual Rev Biochem. 79:507–536.
Xiong H, Carter RA, Leiner IM, Tang Y-W, Chen L, Kreiswirth BN, Pamer EG. 2015. Distinct contributions of neutrophils and CCR2+ monocytes to pulmonary clearance of different *Klebsiella pneumoniae* strains. Infect Immun. 83(9):3418–3427.

Polish Journal of Microbiology
2018, Vol. 67, No 3, 291–295
https://doi.org/10.21307/pjm-2018-034

ORIGINAL PAPER

Evaluation of Modified Hodge Test as a Non-molecular Assay for Accurate Detection of KPC-producing *Klebsiella pneumoniae*

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Submitted 11 October 2017, revised 20 December 2017, accepted 24 March 2018

Abstract

Klebsiella pneumoniae carbapenemase (KPC) have become a major therapeutic challenge because of its increasingly fast dissemination throughout the world. Accurate detection of KPC is essential for optimal treatment. The Clinical and Laboratory Standards Institutes (CLSI) for fast detection of KPC producers currently recommend Modified Hodge Test (MHT) and Carba NP test. MHT can directly detect carbapenemase production in *Enterobacteriaceae* isolates. The current study was conducted to evaluate the capacity of MHT with two carbapenem disks for accurate detection of KPC. MHT was performed according to guidelines of CLSI to identify isolates with carbapenem resistance. In doing so, two substrates of MHT were assigned into two groups for examination: meropenem and ertapenem groups. A total of 96 non-repetitive clinical isolates of *Klebsiella pneumoniae* were tested. The presence of the bla_{KPC} gene in each MHT-positive isolate was examined by PCR. A total of 54 isolates exhibited reduced susceptibility or resistance to carbapenems. Sensitivity of MHT with two carbapenem disks was similar. Specificity of the MHT with meropenem disk was 64% and with ertapenem disk was 53%. Detection of KPC by MHT with meropenem disk was found to be more effective than with ertapenem disk. Based on our results, the presence of KPC does not in itself influence the categorization of resistance. Therefore, the use of MHT with ertapenem disk for the rapid detection of KPC among *K. pneumoniae* for infection control should not be recommended.

Key words: *Klebsiella pneumoniae,* carbapenem disks, detection method, Modified Hodge Test, KPC

Introduction

Klebsiella pneumoniae causes serious hospital-acquired infections of the urinary tract, respiratory tract, surgical sites, and the bloodstream and can cause severe diseases, such as pneumonia, sepsis, and bacteremia (Maroncle et al. 2002; Paterson 2006).The resistance of *K. pneumoniae* has significantly increased with the rampant use of beta-lactam antibiotics, such as carbapenems. Carbapenems are often the last treatment option for infections caused by these multidrug-resistant bacteria. Therefore, the major concern is the development of resistance against carbapenems (Nordmann et al. 2011; Barwa and Shaaban 2017).

Klebsiella pneumoniae carbapenemase (KPC) is a class-A β-lactamase, and the most active family of carbapenemases. The development of antibiotic resistance by class-A Ambler enzymes, such as KPC, generally leads to increased cessation in the treatment of infections (Hashemi et al. 2014; Bachman et al. 2015; Barwa and Shaaban 2017). The strains possessing the bla_{KPC} gene have spread worldwide; this has resulted in increased concern for healthcare services worldwide (Woodford et al. 2010). Detection of carbapenemases in *Enterobacteriaceae* is essential to control the development of resistance in this family, particularly in *K. pneumoniae* isolates. The identification of *K. pneumoniae* isolates producing KPC has become a major concern for clinicians (Djahmi et al. 2014).

In recent years, various phenotypic confirmatory tests to detect the presence of carbapenemase enzymes in *Enterobacteriaceae* have been evaluated by testing the growth of such organisms in CHROMagar KPC medium, Chrom ID ESBL medium, Supercarba

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medium and other tests, i.e., Neo sensitabs (Samra et al. 2008; Nordmann et al. 2011; Aliskan et al. 2012; Hansen et al. 2012).

CLSI has recommended the use of MHT and Carba NP tests for carbapenemase detection in *Enterobacteriaceae*. Because of variations in sensitivity and specificity for detection of KPC producers, the identification of such bacteria through these tests is difficult (Vasoo et al. 2013; Shinde et al. 2017). MHT has acceptable sensitivity. It is easy to perform, inexpensive and feasible for practically all-clinical laboratories; however, it lacks specificity (Anderson et al. 2007; Pasteran et al. 2009; Pasteran et al. 2010). MHT was suggested for detection of carbapenemase-producers based on their *in vivo* production of carbapenemases (Galani et al. 2008; Centers for Disease and Prevention 2009; Miriagou et al. 2010).

The purpose of this study was to evaluate the effectiveness of MHT as a phenotypic confirmatory test with 2 different substrates for KPC screening. Additionally, PCR was performed for the detection of bla_{KPC} gene.

Experimental

Materials and Methods

Bacterial collection and Ribotyping analysis. *K. pneumoniae* isolates were collected from 96 consecutive hospital inpatients and/or outpatients admitted to the AL Zahra hospital in Esfahan, Iran between February and June 2016.

PCR amplification was used for PCR-ribotyping based on internal transcribed spacer (ITS). The procedure implemented was as follows. Template DNA for the PCR was prepared from overnight culture of *K. pneumoniae* on nutrient agar (Scharlo, Spain). Five colonies from the overnight culture on TSA medium (Scharlo, Spain) were suspended in 100 µl distilled water. The boiling lysis method was used for DNA extraction. Cell debris was centrifuged at 13684 RCF for three minutes. Supernatants were used as the source of template DNA for amplification. Strain identifications were performed by analysis of the ITS. PCR-ribotyping was performed using PCR Master Mix 2X (Thermo Scientific, Germany) and specific primers (Liu et al. 2008).

Antimicrobial susceptibility testing. The disk diffusion methods using meropenem, ertapenem disks (Rosco, Denmark) were conducted on the basis of CLSI recommendations. *Escherichia coli* ATCC 25922 and *K. pneumoniae* 700603 were used as reference strains for susceptibility testing (CLSI).

Phenotypic confirmation test. In our study all isolates were subjected to MHT, according to the following procedure. Ertapenem disks (10 µg) (Rosco, Denmark), and meropenem disks (10 µg) (Rosco, Denmark) were used for MHT. The MHT indicator organism, *E. coli* ATCC 25922, was suspended in Mueller-Hinton broth (Scharlo, Spain) to obtain a suspension with turbidity of a 0.5 McFarland standard. The suspension was diluted 1:10 and plated on Mueller-Hinton agar (Scharlo, Spain). The carbapenem disk was placed in the centre of plates, and the isolates were streaked from the margin to the central disk by sterile swab. Two isolates were tested per plate. The plates were then incubated at 35°C for 18–20 hours. The production of a clover leaf-like indentation of the *E. coli* ATCC 25922 growth indicated a positive result for MHT (CLSI).

Genotypic confirmation test. All isolates were subjected to PCR to check for the presence of the bla_{KPC} gene. *K. pneumoniae* ATCC BAA-1705 and *K. pneumoniae* ATCC BAA 1706 were used as quality control strains. A commercial DNA plasmid extraction kit was used (IBRC, Iran) to purify and characterize the plasmid DNA derived from the isolates. The primers used for amplification of bla_{KPC} gene were designed in this study. The forward and reverse primers were

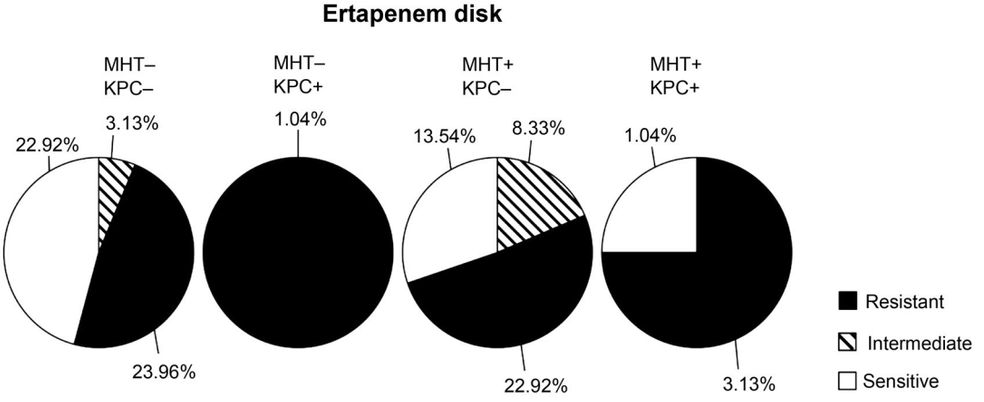

Fig. 1. Result of antimicrobial susceptibility of positive MHT isolates by ertapenem disk. Antimicrobial susceptibility of ertapenem disk and its patterns compared with existence of the bla_{KPC} gene.
Results show that 25% isolates were MHT-negative and resistant to ertapenem disk.

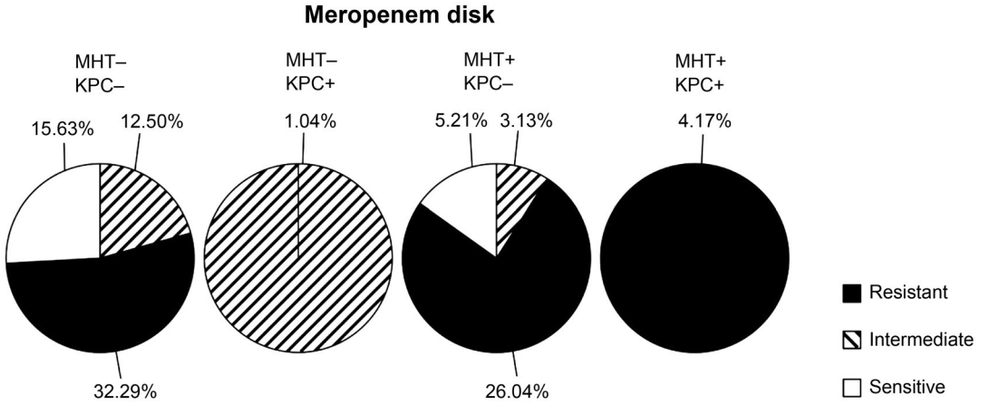

Fig. 2. Result of antimicrobial susceptibility of positive MHT isolates by meropenem disk. Antimicrobial susceptibility of meropenem disk and its patterns compared with existence of the bla_{KPC} gene. Results show that 32.29% isolates were MHT-negative and resistant to meropenem disk.

KPC-F: ATGTCACTGTATCGCCGTCT, and KPC-R: GCTGTGCTTGTCATCCTTGT (Fazabiotech, Iran), respectively. The primers were used at 1 μmol concentration. The amplification product was expected to be 819 bp in length.

PCR reactions were performed using a thermocycler with the following conditions: initial denaturing at 95°C for 5 min, followed by 35 cycles of 60 sec of denaturation at 95°C, annealing at 53°C for 30 sec, elongation at 72°C for 90 sec, and a final extension at 72°C for 10 min.

Results

Bacterial isolation. A total of 96 *K. pneumoniae* were isolated from 96 different patients of whom 15.6% were outpatient and 84.4% were admitted to internal medicine wards. Clinical specimens included 30 urine, 16 wound swabs, one peritoneal-fluid, 6 blood samples, 34 respiratory secretions, and 9 other specimens.

Positive MHT patterns. 37 isolates were classified as carbapenem-resistant using MHT with the meropenem disk, and 47 with the ertapenem disk. A total of 54 isolates (some isolates showed positive result with the use of both disks) were classified as carbapenemase-producer.

Antimicrobial susceptibility patterns. The antimicrobial susceptibility test for two carbapenem antibiotics revealed that 49 isolates (51.04%) were resistant to ertapenem, and 60 isolates (62.5%) were resistant to meropenem.

Antimicrobial susceptibility patterns based on MHT results. 25 percent of the isolates were resistant to ertapenem, whereas they showed negative results by MHT with ertapenem disk (Table I). Only 29 (30.2%) of the MHT-positive isolates with meropenem disk were resistant to meropenem (Table II).

Molecular analysis of bla_{KPC} gene. Five of the 54 isolates, which were carbapenem-resistant and possessed the bla_{KPC} gene, were detected using PCR. Three of these 5 isolates exhibited MHT-positive phenotype with both disks, one with meropenem disk, and one with ertapenem.

Antimicrobial susceptibility of bla_{KPC} gene. The disk diffusion method was interpreted for KPC-producing *K. pneumoniae* isolates as follows: four isolates (4.17%) were resistant to carbapenem disks, two isolates were sensitive to ertapenem disk, and one isolate (1.04%) was intermediate to meropenem disk.

Table I
Result of antimicrobial susceptibility pattern[a] of MHT[b] by ertapenem disk.

		Sensitive	Intermediate	Resistant
MHT	Negative	22.9%	3.1%	25.0%
	Positive	14.6%	8.3%	26.0%

[a] The interpretation was performed based in CLSI guideline (M100-S25). Antimicrobial susceptibility tests were determined using disk diffusion methodology.
[b] MHT positive and negative results were interpreted using the CLSI guideline(M100-S25).

Table II
Result of antimicrobial susceptibility pattern[a] of MHT[b] by meropenem disk.

		Sensitive	Intermediate	Resistant
MHT	Negative	15.6%	13.5%	32.3%
	Positive	5.2%	3.1%	30.2%

[a] The interpretation was performed based in CLSI guideline (M100-S25). Antimicrobial susceptibility tests were determined using disk diffusion methodology.
[b] MHT positive and negative results were interpreted using the CLSI guideline (M100-S25).

Discussion

The rapid detection of carbapenemase-producing strains in clinical samples is critically important to provide appropriate treatment. As a phenotypic test, MHT is widely used for first-line detection of carbapenem resistance in clinical laboratories (Carvalhaes et al. 2010; Cury et al. 2012; Chande et al. 2013). In the current study, we evaluated the efficiency of MHT test with two different carbapenem disks as substrate for identification of KPC enzyme and compared it using PCR.

In this examination, 54 *K. pneumoniae* isolates were MHT-positive, while only five of them were carriers of bla_{KPC} gene. Our finding seems to be consistent with those other studies that obtained false-positive results for MHT. Therefore, the use of MHT alone should not be recommended to confirm the presence of carbapenemase produced by *Enterobacteriaceae* (Bayramoglu et al. 2016)

Varying sensitivity and specificity of MHT were found in different studies (Doyle et al. 2012; Lari et al. 2014; Shinde et al. 2017; Sun et al. 2017). One study revealed that MHT with ertapenem disk could be utilized for the detection of carbapenemases in isolates that showed intermediate or sensitive zone diameter on disk diffusion (Amjad et al. 2011). Another study demonstrated that the MHT with ertapenem disk had positive predictive significance of KPC detection in *Enterobacteriaceae* (Cury et al. 2012).

Our results revealed that the sensitivity of MHT test with meropenem and ertapenem disks was equal (80%). Hence, using two carbapenem disks as a substrate for MHT led to excellent sensitivity for the detecting of KPC producers. The specificity of MHT with ertapenem and meropenem disks was found to be low: 53% and 64%, respectively. However, the specificity of ertapenem disk was much lower than the meropenem disk.

MHT cannot be considered as a good indicator for the detection of KPC producers because of its low specificity. The meropenem disk was more effective than ertapenem disk as a substrate for MHT. Nevertheless, MHT results alone are not sufficient to predict carbapenem resistance. One limitation of the study is that we did not detect other carbapenemase producers. MHT showed carbapenemase activity other than carbapenemase production. The resistance could be related to some other mechanisms. PCR yielded sufficient results for detection of KPC-producing isolates. We recommended that improvement in MHT for the screening of KPC producers with high specificity is required for accurate detection.

Acknowledgements

The authors would like to thank Isfahan science and technology town for this work. No funds have been provided for this study.

Ethical approval

The study was approved by the Ethics Committee of the Alzahra Hospital (Letter number A/120). All microbiological samples were taken as part of standards care procedures. No written informed consent was necessary for this type of study.

Literature

Aliskan HE, Colakoglu S, Turunc T, Demiroglu YZ. 2012. Evaluation of the chromid esbl agar for the detection of esbl-positive *Enterobacteriaceae* and vancomycin-resistant enterococcus isolates from urine cultures. Mikrobiyol Bul. 46(1):17–25.

Amjad A, Mirza I, Abbasi S, Farwa U, Malik N, Zia F. 2011. Modified hodge test: A simple and effective test for detection of carbapenemase production. Iran J Microbiol. 3(4):189–193.

Anderson KF, Lonsway DR, Rasheed JK, Biddle J, Jensen B, McDougal LK, Carey RB, Thompson A, Stocker S, Limbago B et al. 2007. Evaluation of methods to identify the *Klebsiella pneumoniae* carbapenemase in *Enterobacteriaceae*. J Clin Microbiol. 45(8): 2723–2725.

Bachman MA, Breen P, Deornellas V, Mu Q, Zhao L, Wu W, Cavalcoli JD, Mobley HL. 2015. Genome-wide identification of *Klebsiella pneumoniae* fitness genes during lung infection. MBio. 6(3):e00775.

Barwa R, Shaaban M. 2017. Molecular characterization of *Klebsiella pneumoniae* clinical isolates with elevated resistance to carbapenems. Open Microbiol J. 11:152–159.

Bayramoglu G, Ulucam G, Gencoglu Ozgur C, Kilic AO, Aydin F. 2016. Comparison of the modified hodge test and the Carba NP test for detection of carbapenemases in *Enterobacteriaceae* isolates. Mikrobiyol Bul. 50(1):1–10.

Carvalhaes CG, Picao RC, Nicoletti AG, Xavier DE, Gales AC. 2010. Cloverleaf test (modified hodge test) for detecting carbapenemase production in *Klebsiella pneumoniae*: Be aware of false positive results. J Antimicrob Chemother. 65(2):249–251.

Centers for Disease Control and Prevention. 2009. Guidance for control of infections with carbapenem-resistant or carbapenemase-producing *Enterobacteriaceae* in acute care facilities. MMWR Morb Mortal Wkly Rep. 58(10):256–260.

Chande C, Veer P, Chivate A, Joshi SG, Chowdhary A. 2013. What should be the criteria for application of modified Hodge test for carbapenemases in *Klebsiella pneumoniae*? Indian J Med Microbiol. 31(3):318–319.

CLSI CaLSI. 2016. Performance standards for antimicrobial susceptibility testing; twenty-sixth informational supplement. Wayne, PA.

Cury AP, Andreazzi D, Maffucci M, Caiaffa-Junior HH, Rossi F. 2012. The modified Hodge test is a useful tool for ruling out *Klebsiella pneumoniae* carbapenemase. Clinics (Sao Paulo). 67(12): 1427–1431.

Djahmi N, Dunyach-Remy C, Pantel A, Dekhil M, Sotto A, Lavigne JP. 2014. Epidemiology of carbapenemase-producing *Enterobacteriaceae* and *Acinetobacter baumannii* in Mediterranean countries. Biomed Res Int. 2014:305784.

Doyle D, Peirano G, Lascols C, Lloyd T, Church DL, Pitout JD. 2012. Laboratory detection of *Enterobacteriaceae* that produce carbapenemases. J Clin Microbiol. 50(12):3877–3880.

Galani I, Rekatsina PD, Hatzaki D, Plachouras D, Souli M, Giamarellou H. 2008. Evaluation of different laboratory tests for the detection of metallo-beta-lactamase production in *Enterobacteriaceae*. J Antimicrob Chemother. 61(3):548–553.

Hansen F, Hammerum AM, Skov RL, Giske CG, Sundsfjord A, Samuelsen O. 2012. Evaluation of rosco neo-sensitabs for phenotypic detection and subgrouping of esbl-, ampc- and carbapenemase-producing *Enterobacteriaceae*. APMIS. 120(9):724–732.

Hashemi A, Fallah F, Erfanimanesh S, Hamedani P, Alimehr S, Goudarzi H. 2014. Detection of beta-lactamases and outer membrane porins among *Klebsiella pneumoniae* strains isolated in Iran. Scientifica (Cairo). 2014:726179.

Lari AR, Azimi L, Rahbar M, Alaghehbandan R, Sattarzadeh-Tabrizi M. 2014. First report of *Klebsiella pneumoniae* carbapenemase-producing *Pseudomonas aeruginosa* isolated from burn patients in Iran: Phenotypic and genotypic methods. GMS Hyg Infect Control. 9(1):Doc06.

Liu Y, Liu C, Zheng W, Zhang X, Yu J, Gao Q, Hou Y, Huang X. 2008. PCR detection of *Klebsiella pneumoniae* in infant formula based on 16s-23s internal transcribed spacer. Int J Food Microbiol. 125(3):230–235.

Maroncle N, Balestrino D, Rich C, Forestier C. 2002. Identification of *Klebsiella pneumoniae* genes involved in intestinal colonization and adhesion using signature-tagged mutagenesis. Infect Immun. 70(8):4729–4734.

Miriagou V, Cornaglia G, Edelstein M, Galani I, Giske CG, Gniadkowski M, Malamou-Lada E, Martinez-Martinez L, Navarro F, Nordmann P et al. 2010. Acquired carbapenemases in gram-negative bacterial pathogens: Detection and surveillance issues. Clin Microbiol Infect. 16(2):112–122.

Nordmann P, Naas T, Poirel L. 2011. Global spread of carbapenemase-producing *Enterobacteriaceae*. Emerg Infect Dis. 17(10): 1791–1798.

Pasteran F, Mendez T, Guerriero L, Rapoport M, Corso A. 2009. Sensitive screening tests for suspected class a carbapenemase production in species of *Enterobacteriaceae*. J Clin Microbiol. 47(6):1631–1639.

Pasteran F, Mendez T, Rapoport M, Guerriero L, Corso A. 2010. Controlling false-positive results obtained with the Hodge and Masuda assays for detection of class a carbapenemase in species of *Enterobacteriaceae* by incorporating boronic acid. J Clin Microbiol. 48(4):1323–1332.

Paterson DL. 2006. Resistance in gram-negative bacteria: *Enterobacteriaceae*. Am J Infect Control. 34(5 Suppl 1):S20–28; discussion S64–73.

Samra Z, Bahar J, Madar-Shapiro L, Aziz N, Israel S, Bishara J. 2008. Evaluation of Chromagar KPC for rapid detection of carbapenem-resistant *Enterobacteriaceae*. J Clin Microbiol. 46(9):3110–3111.

Shinde S, Gupta R, Raut SS, Nataraj G, Mehta PR. 2017. Carba NP as a simpler, rapid, cost-effective, and a more sensitive alternative to other phenotypic tests for detection of carbapenem resistance in routine diagnostic laboratories. J Lab Physicians. 9(2):100–103.

Sun K, Xu X, Yan J, Zhang L. 2017. Evaluation of six phenotypic methods for the detection of carbapenemases in Gram-negative bacteria with characterized resistance mechanisms. Ann Lab Med. 37(4):305–312.

Vasoo S, Cunningham SA, Kohner PC, Simner PJ, Mandrekar JN, Lolans K, Hayden MK, Patel R. 2013. Comparison of a novel, rapid chromogenic biochemical assay, the Carba NP test, with the modified Hodge test for detection of carbapenemase-producing Gram-negative bacilli. J Clin Microbiol. 51(9):3097–3101.

Woodford N, Eastaway AT, Ford M, Leanord A, Keane C, Quayle RM, Steer JA, Zhang J, Livermore DM. 2010. Comparison of BD Phoenix, Vitek 2, and Microscan automated systems for detection and inference of mechanisms responsible for carbapenem resistance in *Enterobacteriaceae*. J Clin Microbiol. 48(8):2999–3002.

Polish Journal of Microbiology
2018, Vol. 67, No 3, 297–305
https://doi.org/10.21307/pjm-2018-035

ORIGINAL PAPER

Optimization of Mixed Solid-state Fermentation of Soybean Meal by *Lactobacillus* Species and *Clostridium butyricum*

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Submitted 27 November 2017, revised and accepted 20 April 2018

Abstract

Soybean meal is the main vegetable protein source in animal feed. Soybean meal contains several anti-nutritional factors, which directly affect digestion and absorption of soy protein, thereby reducing growth performance and value in animals. Fermented soybean meal is rich in probiotics and functional metabolites, which facilitates soybean protein digestion, absorption and utilization in piglets. However, the mixed solid-state fermentation (SSF) conditions of soybean meal remain to be optimized. In this study, we investigated the optimal parameters for SSF of soybean meal by *Lactobacillus* species and *Clostridium butyricum*. The results showed that two days of fermentation was sufficient to increase the viable count of bacteria, lactic acid levels and degradation of soybean protein in fermented soybean meal at the initial moisture content of 50%. The pH value, lowering sugar content and oligosaccharides in fermented soybean meal, was significantly reduced at the initial moisture content of 50% after two days of fermentation. Furthermore, the exogenous proteases used in combination with probiotics supplementation were further able to enhance the viable count of bacteria, degradation of soybean protein and lactic acid level in the fermented soybean meal. In addition, the pH value and sugar content in fermented soybean meal were considerably reduced in the presence of both proteases and probiotics. Furthermore, the fermented soybean meal also showed antibacterial activity against *Staphylococcus aureus* and *Escherichia coli*. These results together suggest that supplementation of both proteases and probiotics in SSF improves the nutritional value of fermented soybean meal and this is suitable as a protein source in animal feed.

Key words: probiotics, proteases, soybean meal, solid-state fermentation

Introduction

The dietary protein source of animal feeds directly affects nutrient utilization and subsequent growth performance in weaning animals, particularly in piglets. Soybean meal is the main source of vegetable protein in animal feed due to its low cost and high nutritional value (Cromwell 2012). However, soybean meal contains several anti-nutritional factors, such as trypsin inhibitor, lectin, α-amylase inhibiting factor and soybean antigens (Grant 1989). These anti-nutritional factors attenuate the nutritional value, utilization and digestibility of soybean protein, leading to digestive and metabolic diseases in animals (Li et al. 1990; Herkelman et al. 1992; Zhao et al. 2008).

Probiotics are live microorganisms, which can confer a health benefit for the host when administered in appropriate and regular quantities (Chaucheyras-Durand and Durand 2010). Previous studies have demonstrated that the soybean meal fermented by probiotics can increase nutrient digestibility, reduce diarrhea and improve growth performance in piglets (Kiers et al. 2003; Hong et al. 2004; Mukherjee et al. 2016). Microbial fermentation of soybean meal efficiently eliminates anti-nutritional factors and enhances nutrient value by producing proteolytic enzymes (Hong et al. 2004). Currently, *Aspergillus* species are widely used to produce fermented soybean meal due to their capacity to produce multiple hydrolysis enzymes (Pinto et al. 2001; Mathivanan et al. 2006; Feng et al. 2007). In addition

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to fungi-based fermentation, *Lactobacillus* species, particularly *Lactobacillus plantarum*, is another facultative anaerobic bacterial strain, which has also been used to ferment soybean meal (Amadou et al. 2010a, 2010b). *L. plantarum* produces lactic acid and removes trypsin inhibitor contents during fermentation, thereby increasing protein hydrolysis and liberating free amino acids (Amadou et al. 2010a, 2010b).

Other lactic acid-producing *Lactobacillus* species, including *Lactobacillus acidophilus*, *Lactobacillus delbrueckii* and *Lactobacillus salivarius* also showed beneficial effects on animals (Dumbrepatil et al. 2008; Deng et al. 2012; De Cesare et al. 2017). In addition, *Clostridium butyricum* is an anaerobic endospore-forming probiotic and has the potential to improve growth performance and immune function in animals (Yang et al. 2012; Zhang et al. 2014). Since different bacteria strains have different enzyme activities, their capacity to degrade anti-nutritional factors and synthesize different metabolites may vary. It has also been reported that the exogenous enzyme supplementation enhances the degradation of soybean protein and inactive anti-nutritional factors in the SSF of soybean meal (Ma and Wang, 2010; Amadou et al. 2011). However, to our knowledge, there have been no reports about the effects of multistrains, such as *L. acidophilus*, *L. delbrueckii*, *L. salivarius* and *C. butyricum* on SSF of soybean meal. Further, the available data concerning the interaction between exogenous proteases and probiotics in SSF of soybean meal is still scarce.

Thus, the purpose of this study was to investigate the optimal parameters of mixed SSF of soybean meal by *Lactobacillus* species and *C. butyricum* and evaluate the nutritional value in fermented soybean meal. The results provide valuable information about the fermentation of soybean meal by multiple strains and are suitable for use on an industrial scale to produce large amounts of functional fermented products.

Experimental

Materials and Methods

Microorganisms and culture conditions. *L. acidophilus* (BCRC 10695), *L. delbrueckii* (BCRC 10696), and *L. salivarius* (BCRC12574) were purchased from the Food Industry Research and Development Institute (Hsinchu, Taiwan). *C. butyricum* MIYAIRI 588 was purchased from Miyarisan Pharmaceutical (Tokyo, Japan). *Staphylococcus aureus* (BCRC 10780) was provided by the Department of Food Science, National Ilan University (Ilan, Taiwan). *Escherichia coli* DH5α was provided by the Department of Animal Science, National Taiwan University (Taipei, Taiwan). After thawing, *L. acidophilus*, *L. delbrueckii* and *L. salivarius* were inoculated into an Erlenmeyer flask containing De Man, Rogosa and Sharpe broth (MRS; Sigma-Aldrich, St. Louis, MO, USA) at 37°C for 24 hours with shaking. *C. butyricum* was inoculated into an Erlenmeyer flask containing brain heart infusion broth (BHI; Sigma-Aldrich, St. Louis, MO, USA) and incubated at 37°C for 48 hours with shaking. *S. aureus* and *E. coli* were grown in Luria-Bertani broth (LB; Sigma-Aldrich, St. Louis, MO, USA) at 37°C for 18 hours with shaking.

Solid-state fermentation. The procured substrates including soybean meal and molasses were ground to fine powder. Each substrate was mixed with water to give the required relative moisture content in a space bag and autoclaved at 121°C for 30 min. The cooled substrates were inoculated with 3% (v/w) inoculums containing *L. acidophilus*, *L. delbrueckii*, *L. salivarius* and *C. butyricum*, mixed carefully under sterile conditions and incubated in a chamber at 37°C. To study the effects of initial moisture content and fermentation duration, fermentations were performed with different initial moisture contents (40, 45 and 50%, w/w) and different fermentation durations (2–6 days). To study the effects of exogenous protease on SSF, acid protease (0.37%, w/w) in powder form with the activity of 50 000 IU/g (Life Rainbow Biotech, Taiwan) was added at the initial moisture content of 50%. Different fermentation durations (12–48 hours) were employed to study their effects on fermented soybean meal. All experiments were performed 3 times. After fermentation, samples of fermented soybean meal were dried at 50°C for 12 hours and homogenized by mechanical agitation. The fermented powder was then stored at 4°C prior to analysis.

Determination of CFU and spore production. Fermented soybean meal was diluted serially in 0.85% NaCl and then plated on selective media for microbial enumeration. MRS agar was used for determination of *Lactobacillus* species and BHI agar was used for determination of *C. butyricum*. Bacterial colonies counts were statistically analyzed and expressed as colony forming units per gram (CFU/g). For determination of the number of spores, fermented soybean meal was diluted in 0.85% NaCl and then heated at 80°C for 10 min before plating on agar plates. After incubation at 37°C for 18 h, the colonies were counted and expressed as colony forming units per gram (CFU/g).

Determination of lactic acid, pH and residual reducing sugars. The pH level of the fermentation products was measured with the aid of a portable digital pH meter (Mettler Toledo, Greifensee, Switzerland). The content of residual reducing sugars in the supernatants of fermentation products was analyzed by a dinitrosalicylic acid method (Miller 1959), and lactic acid levels were determined by HPLC.

Determination of antimicrobial activity. Antimicrobial activity of fermented soybean meal was analyzed using an agar-well diffusion assay. The *S. aureus* BCRC10780 and *E. coli* DH5α were used as indicator organisms for the determination of antimicrobial activity. Fermented soybean meal was diluted in 0.85% NaCl and transferred into a well in the lysogeny broth agar (LB agar; Sigma-Aldrich, St. Louis, MO, USA) containing *S. aureus* BCRC10780 or *E. coli* DH5α. The plates were incubated at 37°C for 24 h and then examined for zones of inhibition. The control discs were impregnated with ampicillin.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The crude protein extracts from fermented soybean meal were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. After electrophoresis, the gel was stained with Coomassie Brilliant Blue R-250 (CBB R-250; Sigma-Aldrich, St. Louis, MO, USA) to visualize total protein contents.

Thin layer chromatography. Sucrose, raffinose and stachyose standards were purchased from Sigma (St. Louis, MO, USA) and dissolved in 80% aqueous ethanol. The fermentation products were dissolved in 80% ethanol and then heated at 70°C for 1 hour. After cooling, the supernatant of the fermentation products was centrifuged at 10 000 rpm for 10 minutes. Standards and samples were applied on a silica gel plate (Merck Silica Gel 60F 254, Darmstadt, Germany) and developed in a propanol, acetic acid, and water mixture (1:1:0.1). The plates were sprayed with α-naphthol, phosphoric acid, and ethanol mixture (1:100:900, g/v/v), followed by heating in an oven to detect saccharides.

Statistical analysis. All experimental data were analyzed by ANOVA using the general linear model (GLM) procedure of SAS (SAS Institute, Cary, NC, USA). Duncan's new multiple range test was used to evaluate the differences between means. P values of less than 0.05 were considered statistically significant.

Results

Optimization of mixed SSF on soybean meal-based medium. Since initial moisture content of fermentation is critical for microorganism growth in SSF, we first investigated the relation between initial moisture content and fermentation duration on soybean fermentation by *Lactobacillus* species (*L. acidophilus*, *L. delbrueckii* and *L. salivarius*) and *C. butyricum*. The results showed that 50% initial moisture had the highest bacterial growth compared with other initial moisture contents (Fig. 1A). Similarly, the effect of different initial moisture content on bacterial growth was observed for different fermentation durations (Fig. 1A). The highest water content of fermented soybean meal was also found at the initial moisture of 50% in SSF (Fig. 1B). The lactic acid levels of fermented soybean meal increased linearly with the increased initial percentage of moisture (Fig. 1C). Consistently, the pH of the fermented soybean meal was negatively correlated with the initial percentage of moisture (Fig. 1D). Furthermore, the reducing sugar content in fermented soybean meal was significantly reduced at the initial moisture of 50% in SSF (Fig. 1E). Similar results were also found for different fermentation durations (Fig. 1E).

We then examined whether the different initial percentage of moisture and fermentation duration have an impact on the degradation of soybean oligosaccharides in SSF. The results showed that soybean oligosaccharides, including raffinose and stachyose were more efficiently degraded at the initial moisture of 50% compared with other initial moisture contents (Fig. 2A). In addition, two days of SSF was sufficient to degrade soybean oligosaccharides compared with four and six days of SSF (Fig. 2A). Furthermore, we investigated whether the different initial percentage of moisture and fermentation duration could affect the degradation of soybean crude protein in SSF. Consistently, two days of fermentation duration in combination with 50% of initial moisture content in SSF was sufficient to degrade soybean crude proteins compared with other conditions (Fig. 2B, indicated by an arrow). After six days of fermentation, degradation of soybean proteins was achieved at almost all initial moisture conditions (Fig. 2B, indicated by an arrow). Taken together, these results demonstrate that the ideal initial percentage of moisture and fermentation duration in mixed SSF of soybean meal by *Lactobacillus* species and *C. butyricum* are initial moisture of 50% and two days of fermentation. Therefore, 50% moisture level was selected as the optimum parameter for the proliferation of multiple strains of probiotics and used for the next optimization process.

Effect of mixed probiotics in combination with protease supplementation on SSF of soybean meal. We further tested whether proteases and probiotics could further improve the nutritional value of soybean meal at the initial moisture of 50% and two days of SSF. The results showed that bacterial growth was further increased in the presence of both proteases and probiotics in SSF of soybean meal from 12 hours to 48 hours of fermentation compared with probiotics supplementation alone (Fig. 3A). The spore production was further elevated in the presence of both proteases and probiotics from 36 to 48 hours of fermentation compared with probiotics alone (Fig. 3B). The additive effect of proteases and probiotics on the water content of fermented soybean was only observed at 48 hours of SSF (Fig. 3C). Probiotics and protease supplementation further increased the lactic acid levels of fermented soybean meal compared with probiotics supplementation alone

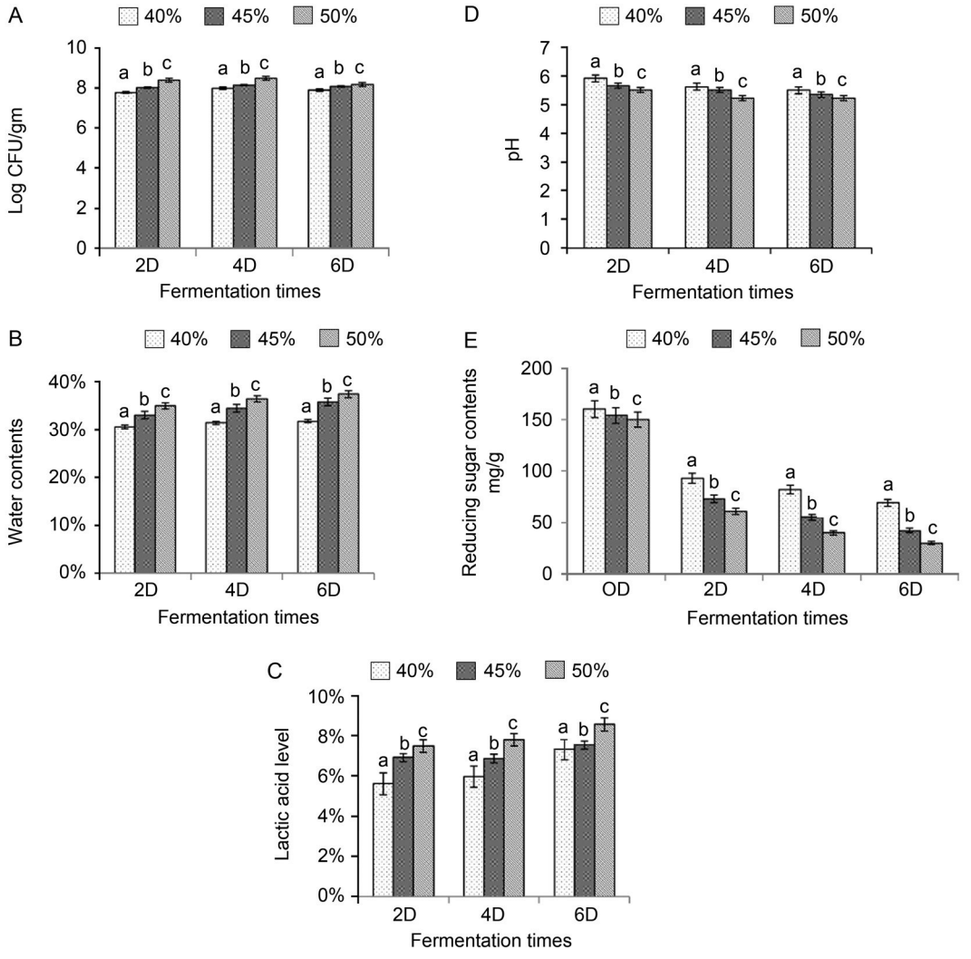

Fig. 1. Effect of different initial moisture and fermentation duration on the soybean meal-based medium in SSF.

(A) Effect of different initial moisture (40%, 45% and 50%) and fermentation duration (2 days; 2D, 4 days; 4D; 6 days; 6D) on colony forming units (CFU) of probiotics in the fermented soybean meal. (B) Effect of different initial moisture (40%, 45% and 50%) and fermentation duration (2 days; 2D, 4 days; 4D; 6 days; 6D) on water contents of the fermented soybean meal. (C) Effect of different initial moisture (40%, 45% and 50%) and fermentation duration (2 days; 2D, 4 days; 4D; 6 days; 6D) on lactic acid level of the fermented soybean meal. (D) Effect of different initial moisture (40%, 45% and 50%) and fermentation duration (2 days; 2D, 4 days; 4D; 6 days; 6D) on pH level of the fermented soybean meal. (E) Effect of different initial moisture (40%, 45% and 50%) and fermentation duration (0 day; 0D, 2 days; 2D, 4 days; 4D; 6 days; 6D) on reducing sugar contents of the fermented soybean meal. Values are expressed as mean ± SD (n = 3). $^{a-c}$Means in the same superscript followed by different letters are significantly different ($p < 0.05$).

(Fig. 3D). The pH of fermented soybean meal was significantly elevated by protease supplementation compared with probiotics supplementation alone, while the pH of fermented soybean meal was further decreased in the presence of both probiotics and protease supplementation (Fig. 3E). Similar to previous results, probiotics supplementation significantly reduced the reducing sugar content in fermented soybean in SSF (Fig. 3F). However, the reducing sugar content was further remarkably decreased in the presence of both probiotics and protease supplementation (Fig. 3F).

We then examined whether proteases and probiotics could affect the degradation of soybean oligosaccharides in SSF. As expected, probiotics supplementation significantly promoted oligosaccharide degradation in fermented soybean meal from 12 to 48 hours of SSF (Fig. 4A). However, protease supplementation did not affect the degradation of soybean oligosaccharide in fermented soybean meal in SSF (Fig. 4A). In addition, supplementation of both proteases and probiotics in SSF did not further increase the degradation of soybean oligosaccharide in fermented soybean meal in SSF (Fig. 4A). Furthermore, we investigated whether supplementation of both proteases and probiotics in SSF could affect the degradation of soybean crude protein in SSF. Similar to previous findings, probiotics supplementation gradually degraded soybean proteins during the fermentation (Fig. 4B, indicated by an arrow), whereas protease supplementation alone did not have a significant effect on the degradation of soybean crude pro-

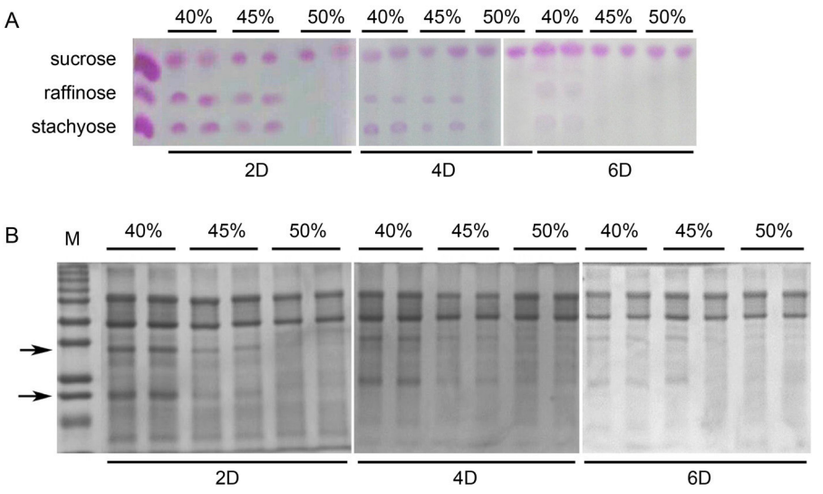

Fig. 2. Effect of different initial moisture content and fermentation duration on soy carbohydrate contents and soy protein contents of the fermented soybean meal.

(A) Effect of different initial moisture (40%, 45% and 50%) and fermentation duration (2 days; 2D, 4 days; 4D; 6 days; 6D) on disaccharide (sucrose) and soy oligosaccharide (raffinose and stachyose) contents of the fermented soybean meal. (B) Effect of different initial moisture (40%, 45% and 50%) and fermentation duration (2 days; 2D, 4 days; 4D; 6 days; 6D) on soy protein of the fermented soybean meal. M represents the standard protein marker. Three experiments were carried out, and one representative result is shown.

teins. Furthermore, the degradation of soybean protein was rapidly achieved in the presence of both proteases and probiotics at the early stage of SSF (Fig. 4B, indicated by an arrow). In addition, we also demonstrated that fermented soybean meal harvested from two days of SSF with supplementation of proteases and probiotics showed antimicrobial activity against *S. aureus* (Fig. 5A) and *E. coli* (Fig. 5B) compared with ampicillin. These results suggest that the supplementation of both proteases and probiotics in SSF is able to improve the nutritional value of fermented soybean meal.

Discussion

In this study, we demonstrated that the optimal initial percentage of moisture and fermentation duration for *L. acidophilus*, *L. delbrueckii*, *L. salivarius*, and *C. butyricum* was 50% and two days in SSF of soybean meal. Supplementation of exogenous protease in combination with probiotics further improved the SSF of soybean meal. Fermented soybean meal was able to inhibit the growth of *S. aureus* and *E. coli* in vitro.

The initial moisture content of dry substrates is a critical factor in the proliferation of bacteria (Zhao et al. 2008). It has been reported that initial moisture content of 50% in SFF increases the growth of *L. plantarum* (Patel et al. 2004). The maximum viable counts of *L. reuteri* was observed at an initial moisture content of 50% in SSF (Zhang et al. 2014). Similarly, we herein found that the maximum viable counts of probiotics were achieved at the initial moisture content of 50% in SSF of soybean meal by *Lactobacillus* species and *C. butyricum*. Moreover, prolonged fermentation duration did not significantly improve bacterial growth in SSF of soybean meal. This result was in accord with the previous studies (Patel et al. 2004; Ying et al. 2009; Zhang et al. 2014). The correlation between initial moisture content, water content and viable counts of bacteria in fermented soybean meal in SSF are relatively less studied. Here, we found that initial moisture content was strictly associated with water content in fermented soybean meal. However, the water content in fermented soybean meal was not positively correlated with viable counts of probiotics, particularly after six days of fermentation. It seems that an optimal range of water content for bacteria growth may occur in SSF of soybean meal. It has been reported that the pH of the fermented product shows a gradual drop in the initial stage of fermentation and then remains steady (Ying et al. 2009; Zhang et al. 2014). In the present study, we used *Lactobacillus* species and *C. butyricum* to ferment the soybean meal and demonstrated that the lactic acid production was linearly increased at the same initial moisture content during the fermentation. The pH value of fermented soybean meal was also consistently decreased at the same initial moisture content after six days of fermentation. These results are similar to the previous findings that lactic acid content increased with increasing initial moisture content during SSF, while the pH value decreased (Dumbrepatil et al. 2008; Ying et al. 2009). Since *C. butyricum* also could reduce pH value in the fermented products by producing butyric acid during fermentation (Zhang et al. 2009), the

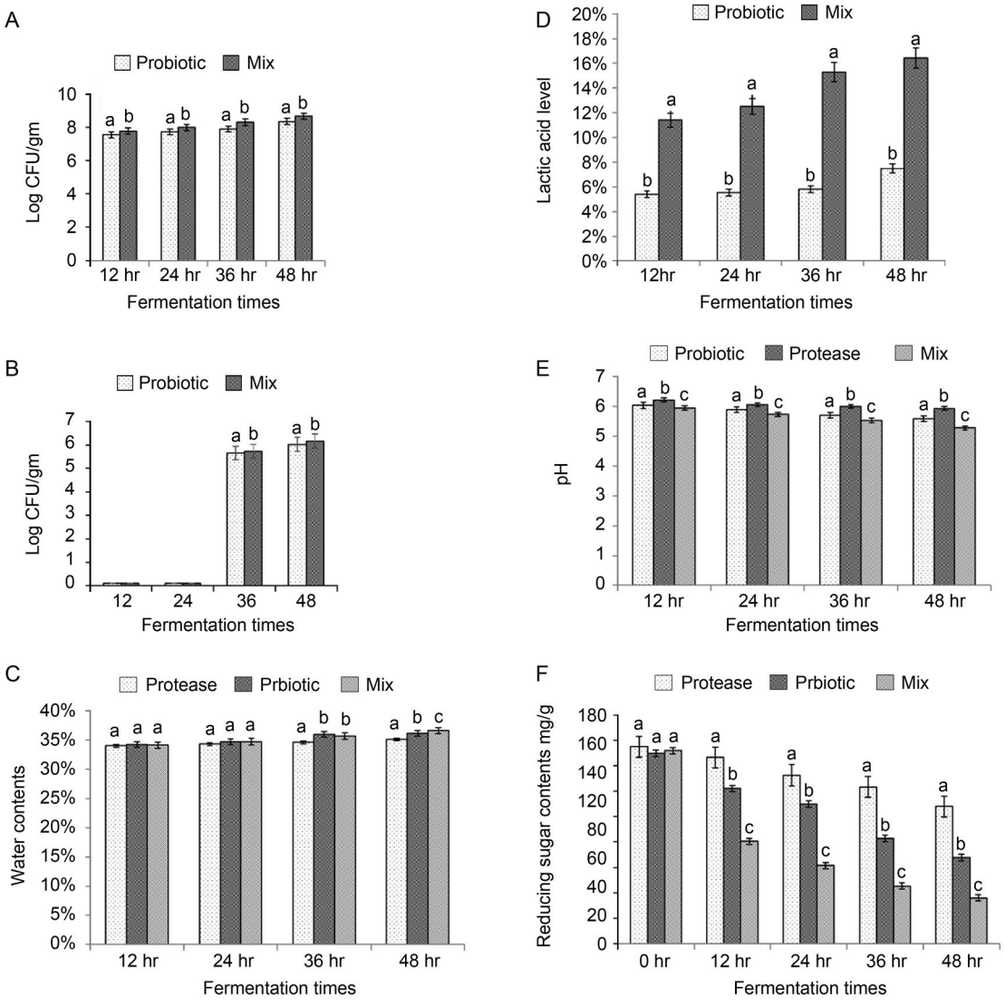

Fig. 3. Effect of different treatments and fermentation duration on the soybean meal-based medium in SSF.

(A) Effect of probiotics (*Lactobacillus* species and *C. butyricum*) and combination (probiotics in combination with proteases), and fermentation duration (12 hours; 12, 24 hours; 24, 36 hours; 36; 48 hours; 48) on colony forming units (CFU) of probiotics of the fermented soybean meal. (B) Effect of probiotics and combination, and fermentation duration (12 hours; 12, 24 hours; 24, 36 hours; 36; 48 hours; 48) on spore production by probiotics of the fermented soybean meal. (C) Effect of probiotics, proteases and combination, and fermentation duration (12 hours; 12, 24 hours; 24, 36 hours; 36; 48 hours; 48) on water contents of the fermented soybean meal. (D) Effect of probiotics, combination and fermentation duration (12 hours; 12, 24 hours; 24, 36 hours; 36; 48 hours; 48) on lactic acid level of the fermented soybean meal. (E) Effect of probiotics, proteases, combination, and fermentation duration (12 hours; 12, 24 hours; 24, 36 hours; 36; 48 hours; 48) on pH level of the fermented soybean meal. (F) Effect of probiotics, proteases and combination, and fermentation duration (12 hours; 12, 24 hours; 24, 36 hours; 36; 48 hours; 48) on reducing sugar contents of the fermented soybean meal. Values are expressed as mean ± SD (n = 3). $^{a-c}$Means in the same superscript followed by different letters are significantly different ($p < 0.05$).

concentration of butyric acid during SSF of soybean meal in the present study remains to be further examined. It has been shown that residual reducing sugar content is negatively associated with viable counts of bacteria in SSF (Zhang et al. 2014). Our findings were generally in line with an earlier report that lower reducing sugar content indicates that the bacteria proliferated more rapidly (Zhang et al. 2014).

Several reports have demonstrated that microbes could produce a variety of enzymes during fermentation and these enzymes could degrade the soybean oligosaccharides and crude proteins (Hong et al. 2004; Adeyemo and Onilude 2014). We also found that soybean oligosaccharides and crude protein content decreased with increasing initial moisture content in SSF, implying that some hydrolytic enzymes were produced by *Lactobacillus* species or *C. butyricum*. It has been demonstrated that protease supplementation significantly improved the nutritional value of soybean meal in SSF by degradation of the large molecular weight of soybean protein (Wang et al. 2014). The proteases are classified into acidic, neutral and alkaline proteinase according to its optimum pH value. Since *Lactobacillus* species are lactic acid-producing probiotics, we then used acidic pro-

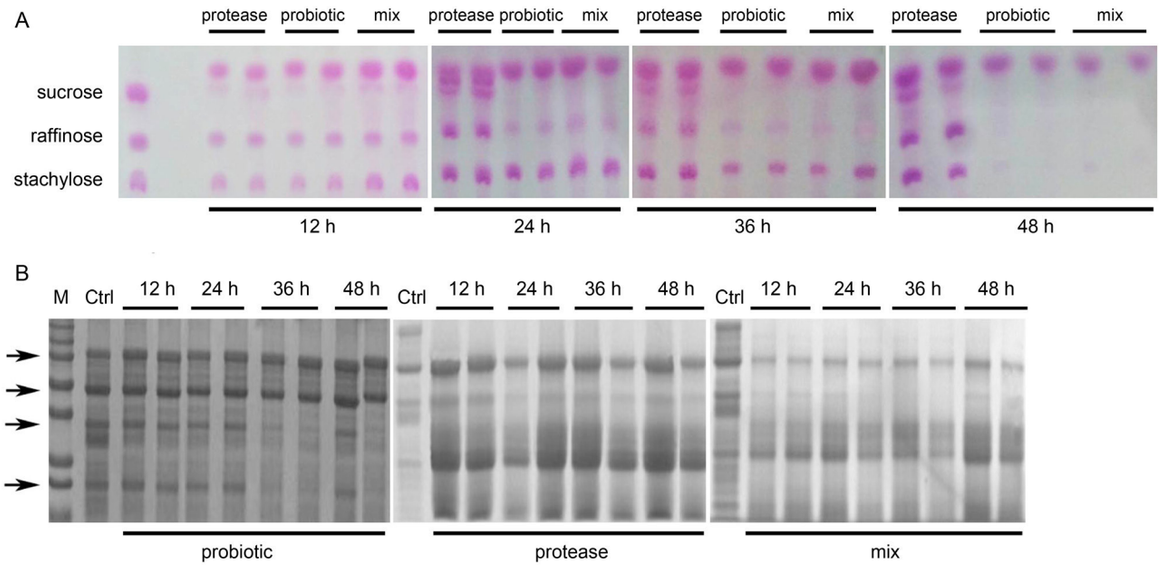

Fig. 4. Effect of different treatments and fermentation duration on soy carbohydrate contents and soy protein contents of the fermented soybean meal.

(A) Effect of different treatments (probiotics, protease and combination) and fermentation duration (12 hours; 12 h, 24 hours; 24 h, 36 hours; 36 h; 48 hours; 48 h) on disaccharide (sucrose) and soy oligosaccharide (raffinose and stachyose) contents of the fermented soybean meal. (B) Effect of different treatments (probiotics, protease and combination) and fermentation duration (12 hours; 12 h, 24 hours; 24 h, 36 hours; 36 h; 48 hours; 48 h) on soy protein of the fermented soybean meal. M represents the standard protein marker. Three experiments were carried out, and one representative result is shown.

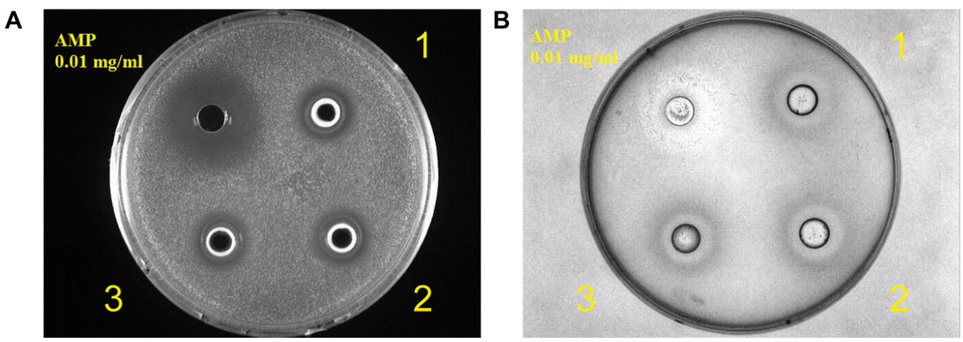

Fig. 5. Assessment of antimicrobial activity of the fermentation product.

(A) Antimicrobial activity of the fermented soybean meal against *S. aureus* compared with ampicillin (AMP). Three experiments were carried out, and one representative result is shown. (B) Antimicrobial activity of the fermented soybean meal against *E. coli* compared with ampicillin (AMP). 1, 2 and 3 represent the antimicrobial results from three independent fermentation experiments.

tease in the present study. Similar to a previous study (Wang et al. 2014), we found that the viable counts of bacteria were significantly increased in the presence of both protease and probiotics. It is possible that amino acids hydrolyzed from soybean proteins by exogenous protease were efficiently utilized by bacteria for growth. Our results including lactic acid production, pH value, residual reducing sugar content and degradation of soybean crude protein supported this hypothesis. Herein, we also found that the spore production of *C. butyricum* was further increased in the presence of both proteases and probiotics in SSF. These results may contribute to the *Lactobacillus* species becoming the dominant strains at the final stage of fermentation and then create a more acidic environment by lactic acid production. Thus, the increased spore production of *C. butyricum* was achieved to resist the harsh environment at the later stages of fermentation. In addition, the more acidic environment caused by *Lactobacillus* species during fermentation also further enhanced the enzyme activity of exogenous acid proteinase. Therefore, the degradation of soybean protein was greatly increased in the presence of both protease and probiotics in SSF compared with supplementation of protease or probiotics alone.

A previous study reported that the pH value was elevated linearly with increased levels of protease supplementation in SSF (Wang et al. 2014). In the present study, we also observed that protease supplementation alone in SSF also consistently increased the pH value.

It has been reported that increased antimicrobial activity of fermented product is mainly due to bacterial metabolites, including acidic substances and bacteriocins (Fuller 1989). Lactic acid from *Lactobacillus* species is able to inhibit the synthesis of bacterial proteins (Wang et al. 2015). *C. butyricum* also can reduce pH value to inhibit the growth of pathogens by producing butyric acid (Zhang et al. 2009). In the current study, the lactic acid production was linearly increased during fermentation and the fermented soybean meal also possessed antimicrobial activity. Thus, it is possible that the antimicrobial activity in the fermented soybean meal might contribute to lactic acid or butyric acid, although the butyric acid production was not measured in our study.

The multistrain probiotics for fermentation have been reported to be more effective than monostrain probiotics, but related research or products are limited (Timmerman et al. 2004; Choi et al. 2011). The previous research reported that the viable counts of *Lactobacillus* species were significantly increased when *Bacillus subtilis* was added to the solid substrate (Zhang et al. 2014). *B. subtilis* followed by *Enterococcus faecium* supplementation effectively improved the quality of corn-soybean meal mixed feed (Shi et al. 2017). Further, fermented soybean meal using multistrain probiotics in combination with protease supplementation improved the nutritional value of soybean meal (Wang et al. 2014). Here, we also demonstrated that SSF by multistrain probiotics also has beneficial effects. Whether the anti-nutritional factors, such as trypsin inhibitor are reduced in SSF and the effects of fermented soybean meal on animals remain to be further investigated.

In conclusion, our results showed that the optimal initial moisture content and fermentation duration for mixed SSF of soybean meal is 50% of initial moisture and two days fermentation. The exogenous protease supplementation in mixed SSF of soybean meal was able to improve the nutritional value of fermented soybean meal.

Acknowledgements

This work was supported by the Ministry of Science and Technology (MOST 105-2622-B-197-001-CC2) in Taiwan.

Literature

Adeyemo SM, Onilude AA. 2014. Reduction of oligosaccharide content of soybeans by the action of *Lactobacillus plantarum* isolated from fermented cereals. Afr J Biotechnol. 13:3790–3796.

Amadou I, Tidjani A, Foh MBK, Kamara MT, Le GW. 2010a. Influence of *Lactobacillus plantarum* Lp6 fermentation on the functional properties of soybean protein meal. Emir J Food Agric. 22: 456–465.

Amadou I, Kamara MT, Tidjani A, Foh MBK, Le GW. 2010b. Physicochemical and nutritional analysis of fermented soybean protein meal by *Lactobacillus plantarum* Lp6. World J Dairy Food Sci. 5:114–118.

Amadou I, Le GW, Shi YH, Gbadamosi OS, Kamara MT, Jin S. 2011. Optimized *Lactobacillus Plantarum* Lp6 solid-state fermentation and proteolytic hydrolysis improve some nutritional attributes of soybean protein meal. J Food Biochem. 35:1686–1694.

Cromwell GL. 2012. Soybean meal: an exceptional protein source. Soybean Meal INFOcenter.
http://www.soymeal.org. accessed 05.09.2012.

Chaucheyras-Durand F, Durand H. 2010. Probiotics in animal nutrition and health. Benef Microbes 1:3–9.

Choi J, Shinde P, Ingale S, Kim J, Kim Y, Kim K, Kwon I, Chae B. 2011. Evaluation of multi-microbe probiotics prepared by submerged liquid or solid substrate fermentation and antibiotics in weaning pigs. Livest Sci. 138:144–151.

Deng J, Li Y, Zhang J, Yang Q. 2012. Co-administration of *Bacillus subtilis* RJGP16 and *Lactobacillus salivarius* B1 strongly enhances the intestinal mucosal immunity of piglets. Res Vet Sci. 94:62–68.

Dumbrepatil A, Adsul M, Chaudhari S, Khire J, Gokhale D. 2008. Utilization of molasses sugar for lactic acid production by *Lactobacillus delbrueckii* subsp. *delbrueckii* mutant Uc-3 in batch fermentation. Appl Environ Microb. 74:333–335.

De Cesare A, Sirri F, Manfreda G, Moniaci P, Giardini A, Zampiga M, Meluzzi A. 2017. Effect of dietary supplementation with *Lactobacillus acidophilus* D2/CSL (CECT 4529) on caecum microbioma and productive performance in broiler chickens. PLoS One. 12(5):e0176309.

Feng J, Liu X, Xu ZR, Liu YY, Lu YP. 2007. Effects of *Aspergillus oryzae* 3.042 fermented soybean meal on growth performance and plasma biochemical parameters in broilers. Anim Feed Sci Technol. 134:235–242.

Fuller R. 1989. Probiotics in man and animals. J Appl Bacteriol. 66:365–378.

Grant G. 1989. Anti-nutritional effects of soyabean: a review. Prog Food Nutr Sci. 13:317–348.

Herkelman KL, Cromwell GL, Stahly TS, Pfeiffer TW, Knabe DA. 1992. Apparent digestibility of amino acids in raw and heated conventional and low-trypsin-inhibitor soybeans for pigs. J Anim Sci. 70:818–826.

Hong KJ, Lee CH, Kim SW. 2004. *Aspergillus oryzae* GB-107 fermentation improves nutritional quality of food soybeans and feed soybean meals. J Med Food. 7:430–435.

Kiers JL, Meijer JC, Nout MJ, Rombouts FM, Nabuurs MJ, van der Meulen J. 2003. Effect of fermented soya beans on diarrhoea and feed efficiency in weaned piglets. J Appl Microbiol. 95:545–552.

Li DF, Nelssen JL, Reddy PG, Blecha F, Hancock JD, Allee GL, Goodband RD, Klemm RD. 1990. Transient hypersensitivity to soybean meal in the early weaned pig. J Anim Sci. 68:1790–1799.

Ma Y, Wang T. 2010. Deactivation of soybean agglutinin by enzymatic and other physical treatments. J Agric Food Chem. 58: 11413–11419.

Miller GL. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Chem. 31:426–428.

Mukherjee R, Chakraborty R, Dutta A. 2016. Role of fermentation in improving nutritional quality of soybean meal – a review. Asian-Australas J Anim Sci. 29:1523–1529.

Mathivanan R, Selvaraj P, Nanjappan K. 2006. Feeding of fermented soybean meal on broiler performance. Int J Poult Sci. 5:868–872.

Patel HM, Wang R, Chandrashekar O, Pandiella SS, Webb C. 2004. Proliferation of *Lactobacillus plantarum* in solid-state fermentation of oats. Biotechnol Prog. 20:110–116.

Pinto GAS, Leite SGF, Terzi SC, Couri C. 2001. Selection of tannase-producing *Aspergillus niger* strains. Braz J Microbiol. 32:24–26.

Shi C, Zhang Y, Lu Z, Wang Y. 2017. Solid-state fermentation of corn-soybean meal mixed feed with *Bacillus subtilis* and *Enterococcus faecium* for degrading antinutritional factors and enhancing nutritional value. J Anim Sci Biotechnol. 8:50.

Timmerman H, Koning C, Mulder L, Rombouts F, Beynen A. 2004. Monostrain, multistrain and multispecies probiotics – a comparison of functionality and efficacy. Int J Food Microbiol. 96:219–233.

Wang Y, Liu XT, Wang HL, Li DF, Piao XS, Lu WQ. 2014. Optimization of processing conditions for solid-state fermented soybean meal and its effects on growth performance and nutrient digestibility of weanling pigs. Livest Sci. 170:91–99.

Wang L, Liu C, Chen M, Ya T, Huang W, Gao P, Zhang H. 2015. A novel *Lactobacillus plantarum* strain P-8 activates beneficial immune response of broiler chickens. Int Immunopharmacol. 29: 901–907.

Yang CM, Cao GT, Ferket PR, Liu TT, Zhou L, Zhang L, Xiao YP, Chen AG. 2012. Effects of probiotic, *Clostridium butyricum*, on growth performance, immune function, and cecal microflora in broiler chickens. Poult Sci. 91:2121–2129.

Ying W, Zhu R, Lu W, Gong L. 2009. A new strategy to apply *Bacillus subtilis* MA139 for the production of solid-state fermentation feed. Lett Appl Microbiol. 49:229–234.

Zhao Y, Qin G, Sun Z, Zhang X, Bao N, Wang T, Zhang B, Zhang B, Zhu D, Sun L. 2008. Disappearance of immunoreactive glycinin and β-conglycinin in the digestive tract of piglets. Arch Anim Nutr. 62:322–330.

Zhao S, Hu N, Huang J, Liang Y, Zhao B. 2008. High-yield spore production from *Bacillus licheniformis* by solid state fermentation. Biotechnol Lett. 30:295–297.

Zhang C, Yang H, Yang F, Ma Y. 2009. Current progress on butyric acid production by fermentation. Curr Microbiol. 59:656–663.

Zhang YR, Xiong HR, Guo XH. 2014. Enhanced viability of *Lactobacillus reuteri* for probiotics production in mixed solid-state fermentation in the presence of *Bacillus subtilis*. Folia Microbiol. 59:31–36.

Zhang L, Cao GT, Zeng XF, Zhou L, Ferket PR, Xiao YP, Chen AG, Yang CM. 2014. Effects of *Clostridium butyricum* on growth performance, immune function, and cecal microflora in broiler chickens challenged with *Escherichia coli* K88. Poult Sci. 93:46–53.

Polish Journal of Microbiology
2018, Vol. 67, No 3, 307–313
https://doi.org/10.21307/pjm-2018-036

ORIGINAL PAPER

Broad Prebiotic Potential of Non-starch Polysaccharides from Oats (*Avena sativa* L.): an *in vitro* Study

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Submitted 6 March 2018, revised 3 April 2018, accepted 20 April 2018

Abstract

Prebiotics inducing the growth or activity of beneficial intestinal bacteria – probiotics producing short-chain fatty acids (SCFA) have lately received wide recognition for their beneficial influence on host intestinal microbiota and metabolic health. Some non-starch polysaccharides (NSP) are defined as prebiotics and oats being one of richest sources of NSP in grains are considered as potentially having prebiotic effect. However, information on fermentation of specific NSP of oats is limited. Moreover, bacterial cross-feeding interactions in which fermentation of prebiotics is involved is poorly characterized. Here, we report the exploration of new candidates for the syntrophic bacterial interactions and fermentability of oat non-starch polysaccharides (NSP). The results obtained by differentiating composition, viscosity and concentration of oats NSP in fermentation medium showed that *Bacillus licheniformis* pre-digests oat NSP, degrades high viscosity of oat β-glucan and makes hemicellulose easier to access for other bacteria. Because of fermentation, *B. licheniformis* produces lactic and succinic acids, which further can be used by other bacteria for cross-feeding and SCFA production.

Key words: Oat NSP, prebiotics, *B. licheniformis, B. ovatus, C. butyricum*

Introduction

Oats are rich source of non-starch polysaccharides (NSP) (4.5 g/100 g); furthermore, 58% of the total NSP are soluble, which is higher than in all the other cereals (Miller and Fulcher 2011). The main component of soluble NSP of oats is (1/3), (1/4)-b-D-glucan (referred as β-glucan). Oats contains 3.2–6.8% β-glucan, which varies with cultivar and environmental effects (Holguin-Acun et al. 2011).

The largest tissue in all of the cereal grains is the endosperm, which may constitute up to 70% of the weight of the mature oat groat (Miller and Fulcher 2011). The fractionation characteristics of isolated endosperm cell wall suggested a layer model: a relatively thin outer layer, consisting of an insoluble polysaccharide skeleton (mostly cellulose, glucomannan, and arabinoxylan) and matrix polysaccharides (β-glucan and arabinoxylan), and also a large inner layer of soluble polysaccharides (mostly β-glucan and small amount of arabinoxylan (Miller and Fulcher 2011).

The cell walls of subaleurone layer of some oat varieties can be very thick. This is an important structural feature allowing production of bran fractions with high β-glucan content (Kaletunc and Breslauer 2003).

Some NSP are defined as prebiotics, which are non-viable food ingredients that can be fermented by specific enzymes derived from gut anaerobic bacteria (Roberfroid 2007). As a result, prebiotics induce the growth or activity of beneficial intestinal bacteria – probiotics, which produce short-chain fatty acids (SCFA). The SCFA are natural ligands for free fatty acid receptor 2 and 3 (FFAR 2/3), found in enteroendocrine and immune cells (Morrison and Preston 2016). Consequently, probiotics and prebiotics are widely used in human and animal nutrition because they beneficially influence the host intestinal microbiota and metabolic health.

Most probiotic strains belong to the genus *Lactobacillus* or *Bifidobacterium*, with only a few belonging to *Enterococcus*, *Escherichia*, or *Streptococcus* (Ulsemer et al. 2012).

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The purpose of the current study was to characterize the syntrophic bacterial interaction and fermentability of both types of oat NSP: with β-glucan (BG25) or without β-glucan (BG0) by less studied bacterial consortia. New candidates for the probiotics: *Bacillus licheniformis* (McFarlin et al. 2017), *Clostridium butyricum* (Kanai et al. 2015) and *Bacteroides ovatus* (Martín et al. 2013) have been explored.

C. butyricum is a common human and animal strictly anaerobe, Gram-positive, gut commensal bacterium frequently found in the environment (Montoya et al. 2001). Some *Clostridium* strains can secrete α- or β-glucanase to degrade polysaccharides (Montoya et al. 2001; Nakajima et al. 2002). Recent studies in animal diabetic models showed positive effects of *C. butyricum* (Sun et al. 2016; Jia, et al. 2017), which could act as a beneficial probiotic for prevention and treatment of hyperglycaemia and associated metabolic dysfunction (Jia et al. 2017).

B. ovatus is also commonly found in mammalian intestine (Rajilić-Stojanović and de Vos 2014), the genus Bacteroides accounts for up to 40% of human colonic microbiota (Hong et al. 2008).

Most recent study discovered a naturally evolved cooperation between *B. ovatus* and *B. vulgatus* within the mammalian intestinal microbiota (Rakoff-Nahoum et al. 2016). A cross-feeding enzymes' system in the gut symbiont *B. ovatus* digests polysaccharides at a cost to itself but at a benefit to another species, such as *B. vulgatus* (Rakoff-Nahoum et al. 2016). Furthermore, recent genetically engineered *B. ovatus* showed a significant prophylactic effect, limiting the development of intestinal inflammation both clinically and histopathologically (Hamady 2013).

In other studies in humans, the probiotic potential of *B. licheniformis* was identified, which was safe and effective for the treatment of diarrhea (Heo et al. 2014). Moreover, a short-term complex probiotic supplementation including *B. licheniformis* spores resulted in decreasing of dietary endotoxemia, the level of triglycerides, and potentially systemic inflammation (McFarlin et al. 2017). However, authors do not explain the nature of these results, where microbiota and SCFA as ligands for FFAR molecules might be involved.

Although it was reported that spores of *B. licheniformis* cause spoilage of milk, dairy products and bread (Pepe et al. 2003; Gopal et al. 2015), the latest study has shown that antimicrobial substances secreted by *B. licheniformis* may serve as useful templates for the development of food preservative agents of natural origin (Arbsuwan et al. 2017).

From the foregoing, it is clear that in the last decade interest in a new probiotic and prebiotic supplementation of human diets has increased due to its co-metabolic interactions between gut microbiota and the host. However, knowledge of the syntrophic fermentation of various oat fractions is still limited, and therefore the present study for the first time considered the fermentability of oat NSP and SCFA production by less studied bacterial consortia.

Experimental

Materials and Methods

Plant Material and Sample Preparation. Oat (*Avena sativa*) flakes were obtained from the "Rigas Dzirnavnieks" Ltd., Latvia. The flakes were milled and sieved through the 0.5 mm sieve. The milled flakes (50 g) were added to glass beaker filled with 500 ml of water (7°C) and mixed by an automatic mixer (Overhead Stirrer Multi Mixer MM-1000, Biosan, Latvia) for 30 min at 120 rpm. The solution was washed through a 160-μm sieve, then residue on sieve was added into beaker filled with 500 ml to repeat washing-sieving process, and to remove any water-soluble compounds (starch, proteins, low molecular weight β-glucan). The residue on sieve was weighted and dried for 4 hours at 60°C in an oven.

To prepare the sample free of β-glucan (BG0), the above-mentioned ready-made 50 g of substrate with a moisture of 7–9%, containing 25% (1,3;1,4)-β-D-glucan (BG25) was dissolved in 200 ml of water. Enzymatic hydrolysis of β-D-glucan to glucose and low molecular weight particles was performed using 0.1% β-glucanase with enzymatic activity ≥ 2 000 000 U/ml (Sunson Industry Group Co. Ltd., China). Hydrolysis was carried out for 90 minutes at 48–52°C, pH 5.3–5.5.

The resulting hydrolysate was cooled to a temperature of 20–25°C. The insoluble fraction of hydrolysate and supernatant containing β-D-glucan were separated by decantation process. The insoluble part of hydrolysate was centrifuged at 5000 rpm for 5 minutes (Thermo Scientific Heraeus X3). The resulting insoluble fraction of substrate was added to glass beaker filled with 500 ml of water (7°C) and washed through a 160 μm sieve, then washing-sieving process was repeated. Residue was dried at a temperature of 45–50°C until moisture of 7–9%. The resulting substrate further was used as BG0 sample.

Both oat substrates were analyzed for β-glucan content, which was determined by the specific enzymatic method (McCleary and Glennie-Holmes 1985) using a mixed-glucan linkage kit (Megazyme Int. Ireland Ltd. Wicklow, Irland).

Viscosity Measurement. Viscosity of the solutions containing 1% β-glucan and 0% β-glucan were measured before and after batch pasteurization process using a digital rotational viscometer equipped with

Pt100 temperature sensor (Thermo Scientific HAAKE Rotational 7 Plus Viscometer). L1 spindle was used to measure dynamic of viscosity. 2.5 grams of BG25 and 2 grams of BG0 were separately mixed with 50 ml of water (20°C) at the room temperature (23°C). Solutions were placed into 100 ml 5 cm diameter glass beakers and gently mixed for 30 seconds, and then heated to 60°C.

Since the viscosity of the solution containing 1% β-glucan varied with its temperature (heating/cooling), different calibration geometries were chosen. After heating it up to 60°C and during its cooling to 40°C 30 rpm/range 200 mPas was chosen. Then, the viscosity reached upper viscosity limit of the selected calibration geometry, the calibration settings were changed to 12 rpm/500 mPas. After pasteurization, calibration parameters of 100 rpm/60 mPas were selected.

To measure the viscosity of solutions containing BG0 prior to the pasteurization process and, the viscosity of solutions containing BG0 and BG25 after the pasteurization process, calibration settings of 100 rpm/60 mPas were selected. All viscosity measurements were conducted in triplicates.

Growth Medium. Bacterial fermentation of oat NSP was conducted in a complex growth medium, which contained 10 g/l beef extract (BBLTM beef extract powder, Becton Dickinson and Co.), 10 g/l peptone (BactoTM peptone, Becton Dickinson and Co.), 5 g/l sodium chloride, 3 g/l sodium citrate, 3 g/l yeast extract (Powdered purified yeast autolysate for bacteriology, Bio-Rad, France), 0.25 g/l L-cysteine (sterile filtered and added after a process of batch pasteurization of the growth medium), all dissolved in distilled water. BG25 as carbon source was added into borosilicate jar with 50 ml growth medium to obtain 1% β-glucan concentration (2.5 g/50 ml). Such growth medium was prepared separately for *C. butyricum* and *B. ovatus*. The same procedure was carried out for the BG0 (2 g/50 ml).

To achieve the required level of sterility, a low temperature batch pasteurization method was used. Tightly sealed bottled samples were pasteurized in a water batch (fryer filled with water) at 80°C for 30 min and then stored at room temperature for repeated continuous process for 7 days.

To determine the level of sterility/contamination of growth medium after pasteurization, 0.1 ml of samples were inoculated on R2A medium (Becton & Dickinson). After 48 hours, bacterial colonies were identified using a commercial biochemical identification kit (BD Diagnostic Systems, Sparks, MD). An aliquot of 1 ml samples of media was transferred to Eppendorf centrifuge tubes and centrifuged at 5000 rpm for 4 minutes (Sigma, Germany). A 0.5 ml of supernatant was taken and diluted with 0.5 ml acetonitrile (ratio 1:1) and frozen at –18°C for further SCFA detection.

The initial pH of the growth medium and pH after 24, 72 and 96 hours of the incubation period of bacteria was measured using a pH meter (AD I 405).

Bacterial Strains and in vitro Fermentation. The bacterial strains used in this study were *C. butyricum* MSCL 1019 and *B. ovatus* MSCL 841, obtained from the Microbial Strain Collection of Latvia, where they were stored in liquid nitrogen. Before use, the strains were cultivated in Wilkins-Chalgren anaerobe agar (Oxoid, UK) under anaerobic conditions (GasPak Anaerobic Pouch, Becton & Dickinson) at 37°C for 48 hours. The harvested strains were adjusted for each growth medium to 4.8×10^5 CFU/ml for *C. butyricum* and 2.2×10^5 CFU/ml for *B. ovatus*. The growth media with bacterial strains were incubated in a thermostat at 37°C. *In vitro* fermentation was carried for 96 hours under anaerobic conditions. The SCFA concentration and pH were measured at 0, 24, 72, 96 hours. Aliquots of 1 mL samples were transferred to Eppendorf centrifuge tubes and centrifuged at 5000 rpm for 4 minutes (Sigma 1-14, Germany). A 0.5 ml of supernatant was taken and diluted with 0.5 ml acetonitrile (ratio 1:1). All samples were done at duplicates and were frozen at –18°C for further SCFA analysis.

SCFA Analysis by LC-TOF-MS. Concentrations of SCFA were determined by liquid chromatography time-of-flight mass spectrometry (LC-TOF-MS) in accordance with previously described analytical methods (Ferrer and Thurman 2003; Ibanez and Bauer 2014; Han et al. 2015). All solvents were of analytical grade. Formic acid was purchased from Sigma-Aldrich (St. Louis, USA). Deionized water (18.2 MΩ) was prepared by a Milli-Q water purification system from Millipore (Billerica, Massachusetts, USA). LC analyses were performed using Agilent 1290 Infinity series (Agilent Technologies, Germany) UHPLC equipped with an Agilent 1290 DAD photodiode array detector. Phenomenex (Torrance, CA) RezexTM ROA-Organic (Acid H+ (8%) (250 mm × 4.6 mm) column was employed for the separation. The column was thermostated at 55 ± 1°C. The injection volume was 2.0 μL.

The mobile phase was directly on-line degassed and its composition consisted of 0.5% (v/v) formic acid in water at a flow rate of 0.30 ml/min in isocratic mode. The high-resolution mass spectra (HRMS) were taken on an Agilent 6230 TOF LC/MS system (Agilent Technologies, Germany) with electrospray ionization (ESI). The source parameters were: negative ionization mode, drying gas flow 10.0 l/min and temperature 285°C, fragmentor ionisation 75 V. One full mass spectrum was acquired in a profile mode, with mass range from m/z 50 to 1100, 1 scan/s. The data of SCFA were obtained using the extraction of individual compound chromatogram at its individual m/z value. The internal reference masses of m/z 112.9856 and m/z 1033.9881

(G1969-85001 ESI-TOF Reference Mass Solution Kit, Agilent Technologies & Supelco) were used for all analyses of the samples. The experimental data were handled using the MassHunter version B07.00 software (Agilent Technologies).

Data Processing and Analysis. Identification of separated SCFA were based on the search for [M-H]- ions, using extracted ion mass chromatograms. The experimentally obtained mass spectra of SCFA approved the calculated values (lactic acid C3H6O3, [M-H]- Calculated – 89.0264, Found – 89.0244, Δ 0.0020; acetic acid C2H4O2, [M-H]-Calculated – 59.0150, Found – 59.0139, Δ 0.0011; succinic acid C4H6O4, [M-H]-Calculated – 117.0217, Found – 117.0193, Δ 0.0024; propionic acid C3H6O2, [M-H]-Calculated – 73.0303, Found – 73.0295, Δ 0.0008; butyric acid C4H8O2, [M-H]-Calculated – 87.0114, Found – 87.0452, Δ 0.0338). For quantification the calibration curves of acetic, propionic, succinic and lactic acids, the standard solutions were constructed by plotting the ratio of the average chromatographic peak area and mass concentration. According to the reflected data, the regression equation of the trend line was calculated. Standard solutions were injected in triplicate, and the corresponding peak areas were recorded. The relative standard deviation between all solutions was determined to be less than 1.5%. The calibration curves obtained showed linearity of the determination coefficient (R2 > 0.99) in the used concentration range (0.5–100 μg/ml). A coefficient of determination (R2) was calculated using Microsoft Excel 2013, $p < 0.001$. Analyses of SCFA measurements done at duplicates were averaged. To compare the means between viscosity measurements one-way analysis of variance (one-way ANOVA) was used, p value of less than 0.05 was considered significant. Data were expressed in μg/ml as means ± S.D.

Results and Discussion

The results of our study showed that the batch pasteurization of solutions with oat NSP did not inactivate the thermostable *B. licheniformis* (biotype validity 18185, confidence 0.9555). Since *B. licheniformis* is a source of β-1,3-1,4-glucanase (Planas 2000) its effect on β-glucan viscosity degradation and SCFA production by the bacteria alone and in consortium with *C. butyricum* and *B. ovatus* was further tested.

The viscosity of a solution containing 1% β-glucan before the pasteurization process, directly after mixing prepared substrate with water (20°C), was 14 ± 1 mPas (pH 6.9). After the solution was heated to 60°C and then cooled to room temperature, there was a tendency to increase the viscosity (Fig. 1).

The final stable viscosity after cooling the solution to room temperature reached 260 mPas (Fig. 1). At the end of the pasteurization process, the viscosity of the solution containing BG25, degraded by *B. licheniformis* decreased to 4 ± 1 mPas and did not differ from the solution containing BG0, in which β-glucan was degraded using commercial enzymes (2 ± 1 mPas vs 4 ± 1 mPas, $p = 0.18$).

Oat β-glucan is able to form highly viscous solutions at low concentrations; however, the viscosity depends on the concentration and the molecular weight of β-glucan (Anttila et al. 2008).

Our results showed that fermentation of oat β-glucan by *B. licheniformis* resulted in degradation of β-glucan viscosity and, consequently, its molecular weight. As noted earlier (Sahasrabudhe et al. 2016), the enzymatic degradation of medium viscosity of oat β-glucan enhances its effect on immune receptors. The digested oat β-glucan activates type II transmembrane protein receptor Dectin-1 that binds β-1,3- and β-1,6-glucans more efficiently than non-digested β-glucan

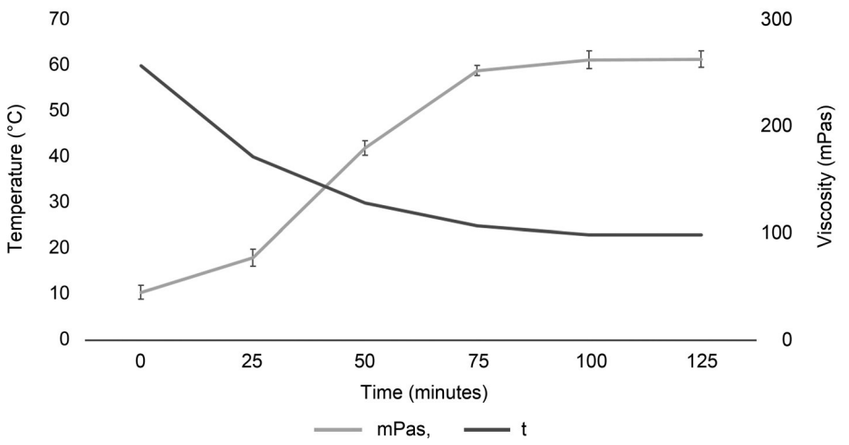

Fig. 1. Changes in the viscosity of a solution containing 1% oat β-glucan, after heating it up to 60°C and cooling to room temperature. Data represent mean ± SD of triplicates.

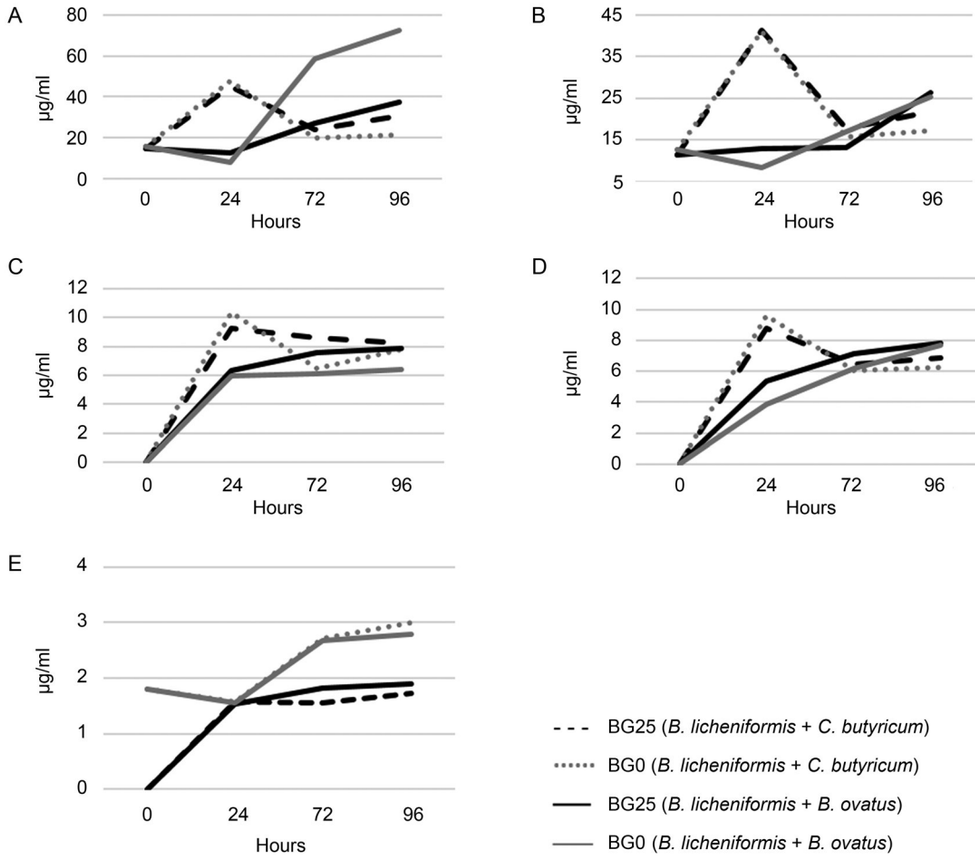

Fig. 2. Changes in concentration of lactic (A), succinic (B), butyric (C), propionic (D), and acetic (E) acids within 96 hours of syntrophic *in vitro* fermentation of BG0 and BG25 by *B. licheniformis* in the consortium with *C. butyricum* and *B. ovatus*.

(Sahasrabudhe et al. 2016). In this way, pre-digestion of high molecular weight of oat β-glucan by *B. licheniformis* may have more visible host's microbial-independent immune modulation effects *in vivo* through the pattern-recognition receptor Dectin-1 activation, what should be explored in future.

After pasteurizing of the growth medium containing *B. licheniformis*, lactic acid was detected in both BG25 (14.8 µg/ml) and BG0 (15.6 µg/ml), and also succinic acid at a concentration of 11.4 µg/ml in BG25 and 12.7 µg/ml in BG0, respectively. Propionic and butyric acids have not been detected in any of the growth media (BG25 and BG0), indicating that *B. licheniformis* does not have the potential to produce these SCFA. A very low concentration of acetic acid (1.8 µg/ml) produced by *B. licheniformis* was detected in BG0.

After *B. ovatus* was added to the growth medium containing *B. licheniformis*, the lactic acid concentration decreased in both BG25 and BG0 after 24 hours (Fig. 2A). Succinic acid decreased in BG0 after 24 hours period, but not in BG25 (Fig. 2B). These data show that *B. ovatus* within the 24 hours of fermentation consumed lactic and succinic acids produced by *B. licheniformis* (Fig. 2A, 2B).

Because *B. licheniformis* produces lactic and succinic acids, it could be involved in bacterial cross-feeding and butyrate synthesis through the butyril-CoA: acetate CoA-transferase pathway (Reichardt et al. 2014), as well as in the synthesis of propionate through either succinate synthesis pathway or acrylate pathway using lactate (Reichardt et al. 2014).

The concentration of lactic acid, produced by *B. licheniformis* in a consortium with *B. ovatus* in BG25 was lower than in BG0 (58.5 vs 27.1 µg/ml after 72 hours and 37.5 vs 72.6 µg/ml after 96 hours of fermentation for BG25 and BG0 (Fig. 2A). One of the hypotheses that explain this finding can be an assumption that degradation of β-glucan by commercial enzymes facilitates access to hemicellulose for bacteria. This hypothesis is also implied by the fact that the pH value in BG0 was lower than in BG25 (Fig. 3A, 3B).

Since the β-xylosidases from *B. ovatus* have been found to be highly effective in digesting hemicellulose to xylooligosaccharides (Jordan et al. 2017), it is

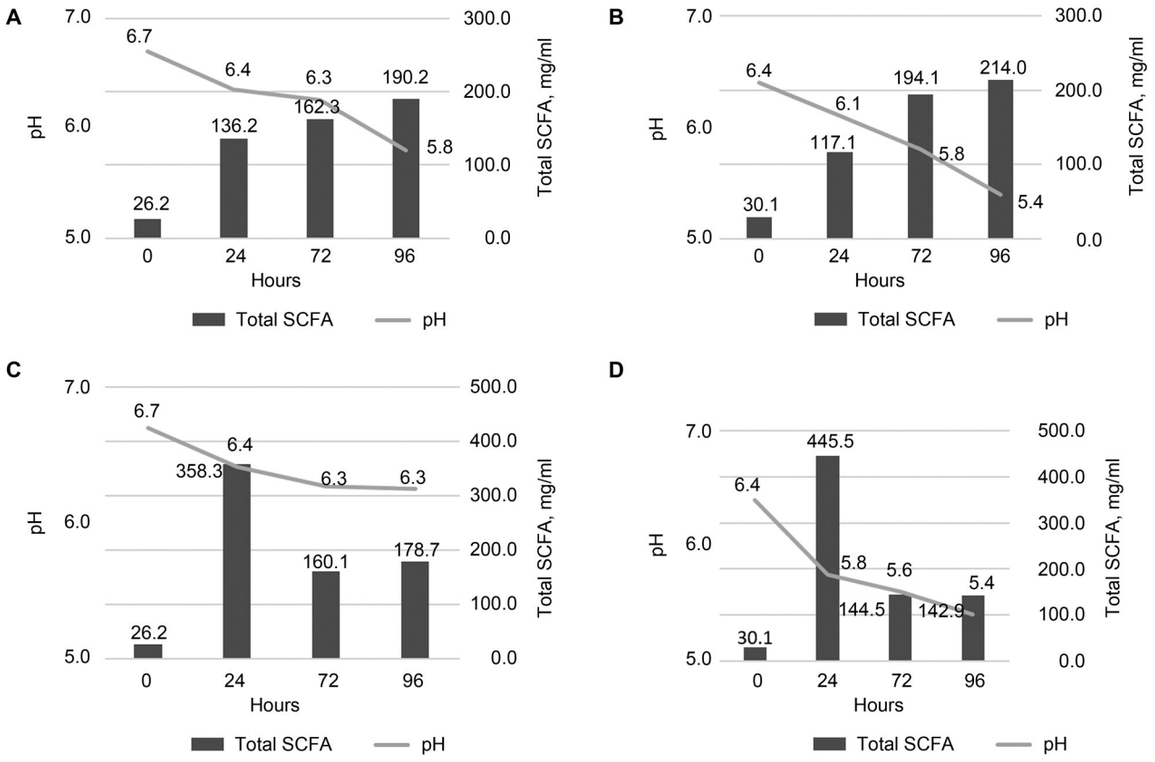

Fig. 3. The total acids concentrations and changes in pH within the 96 hours of syntrophic *in vitro* fermentation of BG25 (A, C) and BG0 (B, D) by *B. ovatus* (A, B) and *C. butyricum* (C, D) in consortium with *B. licheniformis*.

likely that oat hemicelluloses that were not digested by *B. licheniformis,* have been finally degraded by *B. ovatus* into xylooligosaccharides. These xylooligosaccharides can become substrates for *B. licheniformis* through the use of xylanase enzymes. As a result, further syntrophic fermentation of xylooligosaccharides led to the production of lactic and succinic acids by *B. licheniformis*.

The results of our study showed that fermentation of BG0 and BG25 during culture of the consortium consisted of *B. licheniformis* and *B. ovatus* or *C. butyricum* resulted in an increase of the concentration of propionic and butyric acids (Fig. 2C, 2D), while the production of butyrate and propionate by the same bacterium is not common. Only a few anaerobes, such as *Roseburia inulinivorans* and *Coprococcus catus* were previously mentioned having the ability to produce both (Reichardt et al. 2014).

The total acid concentration peak for *B. licheniformis* in consortium with *C. butyricum* was observed after 24 hours of fermentation in BG25 and BG0 (Fig. 3C, 3D). It was assumed that *B. licheniformis* degraded large β-glucan molecules and produced lactic and succinic acids, while the simple or/and complex sugars were degraded by *C. butyricum*, because of their previously mentioned enzymatic capacities (Montoya et al. 2001; Nakajima et al. 2002).

The acetic acid produced by *B. licheniformis* in consortium with *C. butyricum* or *B. ovatus* did not reach 3 μg/ml (Fig. 2E), which is the lowest concentration of all the acids analysed.

Because the current study did not consider antagonistic interactions between members of the bacterial consortium, a further study are needed to investigate if *B. licheniformis* competes for the substrate with other bacteria, and thereby reduces availability of the substrate to another bacterium (pathogenic or probiotic).

Conclusions

In conclusion, the results of this study show that *B. licheniformis*, a widespread food contaminant, acts as thermostable probiotic that ferments oat NSP. It pre-digests oat NSP, degrades the high viscosity of oat β-glucan and makes hemicellulose more accessible for other bacteria. As a result of fermentation, *B. licheniformis* produces lactic and succinic acids, which further can be used by other bacteria for cross-feeding and production of SCFA. Thus, *B. licheniformis* in syntrophic consortia with *C. butyricum* or *B. ovatus* produces SCFA, which were not produced when *B. licheniformis* was grown alone.

Disclosure of interest

The authors report that they have no conflicts of interest.

Literature

Anttila H, Sontag-Strohm T, Salovaara H. 2008. Viscosity of beta-glucan in oat products. AFSci. 13(1–2):80–87.

Arbsuwan N, Payoungkiattikun W, Sirithorn P, Daduang S, Jangpromma N, Dhiravisit A, Tae Hahm Y, Kurt Neubert L, Klaynongsruang S. 2017. Purification and characterization of macrolactins and amicoumacins from *Bacillus licheniformis* BFP011: a new source of food antimicrobial substances. CyTA – Journal of Food. 16(1):50–60.

Ferrer I, Thurman EM. 2003. Liquid chromatography time – of – flight/ mass spectrometry (LC/TOF/MS) for the analysis of emerging contaminants. Trends Anal Chem. 22(10):750–756.

Gopal N, Hill C, Ross PR, Beresford TP, Fenelon MA, Cotter PD. 2015. The prevalence and control of *Bacillus* and related spore-forming bacteria in the dairy industry. Front Microbiol. 21(6):1418.

Hamady ZZR. 2013. Novel xylan-controlled delivery of therapeutic proteins to inflamed colon by the human anaerobic commensal bacterium. Ann R Coll Surg Engl. 95(4):235–240.

Han J, Lin K, Sequeira C, Borchers CH. 2015. An isotope-labeled chemical derivatization method for the quantitation of short-chain fatty acids in human feces by liquid chromatography-tandem mass spectrometry. Anal Chim Acta. 7(854):86–94.

Heo J, Kim SK, Park KS, Jung HK, Kwon JG, Jang BI. 2014. A double-blind, randomized, active drug comparative, parallel-group, multi-center clinical study to evaluate the safety and efficacy of probiotics (*Bacillus licheniformis,* Zhengchangsheng® capsule) in patients with diarrhea. Intest Res. 12(3):236–244.

Holguin-Acun AL, Ramos-Chavira N, Carvajal-Millan E, Santana-Rodriguez V, Rasco´n-Chu A, Niño-Medina G. 2011. Non-starch polysaccharides in maize and oat. In: Preedy VR, Watson RR, Patel VB, editors. Flour and breads and their fortification in health and disease prevention. 1st ed. San Diego, California (USA): Academic Press. p. 542.

Hong PY, Wu JH, Liu WT. 2008. Relative abundance of *Bacteroides* spp. in stools and wastewaters as determined by hierarchical oligonucleotide primer extension. Appl Environ Microbiol. 74(9):2882–2893.

Ibanez AB, Bauer S. 2014. Analytical method for the determination of organic acids in dilute acid pretreated biomass hydrolysate by liquid chromatography-time-of-flight mass spectrometry. Biotechnol Biofuels. 7(1):145.

Jia L, Li D, Feng N, Shamoon M, Sun Z, Ding L, Zhang H, Chen W, Sun J, Chen YQ. 2017. Anti-diabetic effects of *Clostridium butyricum* CGMCC0313.1 through promoting the growth of gut butyrate-producing bacteria in type 2 diabetic mice. Sci Rep. 7(1):7046.

Jordan DB, Stoller JR, Lee CC, Chan VJ, Wagschal K. 2017. Biochemical characterization of a GH43 β-xylosidase from *Bacteroides ovatus.* Appl Biochem Biotechnol. 182(1): 250–260.

Kaletunc G, Breslauer K. 2003. Characterization of cereals and flours. 1st ed. Boca Raton (USA): CRC Press.

Kanai T, Mikami Y, Hayashi A. 2015. A breakthrough in probiotics: *Clostridium butyricum* regulates gut homeostasis and anti-inflammatory response in inflammatory bowel disease. J Gastroenterol. 50(9):928–939.

Martín R, Miquel S, Ulmer J, Kechaou N, Langella P, Bermudez-Humaran LG. 2013. Role of commensal and probiotic bacteria in human health: a focus on inflammatory bowel disease. Microb Cell Fact. 12:71.

McCleary BV, Glennie-Holmes M. 1985. Enzymic quantification of (1→3) (1→4)-β-D-glucan in barley and malt. JIB. 91(5): 285–295.

McFarlin BK, Henning AL, Bowman EM, Gary MA, Carbajal KM. 2017. Oral spore-based probiotic supplementation was associated with reduced incidence of post-prandial dietary endotoxin, triglycerides, and disease risk biomarkers. World J Gastrointest Pathophysiol. 8(3):117–126.

Miller SS, Fulcher RG. 2011. Microstructure and Chemistry of the Oat Kernel. In: Webster FH, Wood PJ, editors. Oats: Chemistry and Technology. 2nd ed. St.Paul, Minnesota (U.S.A.): AACC International. p. 77–94.

Montoya D, Arévalo C, Gonzales S, Aristizabal F, Schwarz WH. 2001. New solvent-producing *Clostridium* sp. strains, hydrolyzing a wide range of polysaccharides, are closely related to *Clostridium butyricum*. J Ind Microbiol Biotech. 27(5):329–335.

Morrison DJ, Preston T. 2016. Formation of short chain fatty acids by the gut microbiota and their impact on human metabolism. Gut Microbes. 7(3):189–200.

Nakajima N, Ishihara K, Matsuura Y. 2002. Dietary-fiber-degrading enzymes from a human intestinal *Clostridium* and their application to oligosaccharide production from nonstarchy polysaccharides using immobilized cells. Appl Microbiol Biotechnol. 59: 82–189.

Pepe O, Blaiotta G, Moshetti G, Greco T, Villani F. 2003. Rope-producing strains of *Bacillus* spp. from wheat bread and strategy for their control by lactic acid bacteria. Appl Environ Microbiol. 69(4):2321–2329.

Planas A. 2000. Bacterial 1,3-1,4-beta-glucanases: structure, function and protein engineering. Biochim Biophys Acta. 1543(2):361–382.

Rajilić-Stojanović M, de Vos W. 2014. The first 1000 cultured species of the human gastrointestinal microbiota. FEMS Microbiol Rev. 38(5):996–1047.

Rakoff-Nahoum S, Foster K, Comstock L. 2016. The evolution of cooperation within the gut microbiota. Nature. 533(7602):255–259.

Reichardt N, Duncan SH, Young P, Belenguer A, McWilliam Leitch C, Scott KP, Flint HJ, Louis P. 2014. Phylogenetic distribution of three pathways for propionate production within the human gut microbiota. ISME J. 8(6):1323–1335.

Roberfroid M. 2007. Prebiotics: the concept revisited. J Nutr. 137(3 Suppl 2):830S–837S.

Sahasrabudhe NM, Tian L, van den Berg M, Bruggeman G, Bruininx E, Schols HA, Faas MM, de Vos P. 2016. Endo-glucanase digestion of oat beta-glucan enhances Dectin-1 activation in human dendritic cells. J Funct Foods. 21:104–112.

Sun J, Wang F, Ling Z, Yu X, Chen W, Li H, Jin J, Pang M, Zhang H, Yu J, Liu J. 2016. *Clostridium butyricum* attenuates cerebral ischemia/reperfusion injury in diabetic mice via modulation of gut microbiota. Brain Res. 1642:180–188.

Ulsemer P, Toutounian K, Schmidt J, Karsten U, Goletz S. 2012. Preliminary safety evaluation of a new *Bacteroides xylanisolvens* isolate. Appl Environ Microbiol. 78(2):528–535.

Polish Journal of Microbiology
2018, Vol. 67, No 3, 315–320
https://doi.org/10.21307/pjm-2018-037

ORIGINAL PAPER

The Effect of Silver Nanoparticles on *Listeria monocytogenes* PCM2191 Peptidoglycan Metabolism and Cell Permeability

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Submitted 11 January 2018, revised 18 February 2018, accepted 10 May 2018

Abstract

Listeria monocytogenes is Gram-positive bacterial pathogen, a causative agent of food poisoning and systemic disease – listeriosis. This species is still susceptible to several conventionally used antibiotics but an increase in its resistance has been reported. For this reason the search for new, alternative therapies is an urgent task. Silver nanoparticles seem to be the promising antibacterial agent. Minimal inhibitory concentration of silver nanoparticles was determined. Sublethal concentrations were used in study of nanosilver effect on cells lysis by estimation of the number of cells surviving the treatment with 0.25 or 0.5 of minimal inhibitory concentrations of silver nanoparticles. Autolysis of isolated peptidoglycan was studied by measuring the absorbance of preparation subjected to nanosilver treatment. Silver nanoparticles effect on *L. monocytogenes* envelopes permeability was determined by measuring the efflux of cF, DNA and proteins. It was demonstrated that nanosilver enhanced the lysis of *L. monocytogenes* cells and, to the lesser extent, autolysis of isolated peptidoglycan. The increase in the efflux of carboxyfluoresceine, DNA and proteins was also noted. The obtained results allow to postulate that *L. monocytogenes* peptidoglycan, constituting the main component of cell wall, is the target of silver nanoparticles activity against this pathogen.

Key words: *Listeria monocytogenes*, autolysis, peptidoglycan, permeability, silver nanoparticles

Introduction

An ionic form of silver has been used for centuries to cure several diseases which causative agents were bacteria such as *Staphylococcus aureus*, *Klebsiella* sp. and *Pseudomonas* sp. (Rai et al. 2009). It was shown that silver nanoparticles, AgNPs, have higher antibacterial activity than silver ions (Ingle et al. 2008). Nanoparticles are defined as the clusters of atoms of size from 1 to 10 nm with a large surface area to volume ratio, what is proportionally correlated with AgNPs antibacterial activity (Morones et al. 2005). Several studies demonstrated that antibacterial effect of silver nanoparticles is based on their interaction and subsequent damage of cell membranes and on the induction of reactive oxygen species (ROS), including free radicals. The interaction of AgNPs with cell membranes is promoted by their strongly positive zeta potential, which is a difference of an electric potential between the particle and the surrounding solution (Stapsford et al. 2011). This interaction leads to membrane disruption, bacterial flocculation, efflux of cytoplasm, and as a consequence reduction in viability. Formation of ROS is responsible for oxidation, subsequent inactivation and damage of cellular proteins and DNA and peroxidation of lipids (Singh et al. 2008).

It was also proved that AgNPs are active against bacterial biofilms, which are complex bacterial communities resistant to antibiotics and the human immune system. Biofilm resistance is very important and now constitutes a medical challenge as recently the number of infections associated with antibiotic-resistant bacteria living in biofilms has been increased exponentially. These included infections caused by *Pseudomonas aeruginosa*, the causative agent of nosocomial respiratory tract pneumonia, infections of burn wounds, and chronic lung infections of patients with cystic fibrosis. Biofilm formed by *S. aureus* also constitutes a very important clinical problem being

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responsible of e.g. osteomyelitis, periodontitis and chronic wound infections. Well-defined diseases are also caused by biofilms formed by gastrointestinal tract pathogens belonging to Enterobacteriaceae family (for review see Markowska et al. 2013; Wolska et al. 2015). Beside their intrinsic antibacterial activity silver nanoparticles were proved to enhance the effect of conventional antibiotics, such as: vancomycin, amoxicillin, gentamycin, ampicillin, streptomycin in curing bacterial infections (Shahverdi et al. 2007; Birla et al. 2009).

Listeria monocytogenes is a Gram-positive pathogen able to survive in a variety of environments including food, soil and humans. It constitutes very severe problem for food industry because it can survive and multiply even in low temperature; moreover, it forms biofilm and therefore is hard to be eradicated from food production lines. This species is characterized by a unique life mode; it grows in the cytoplasm of the host cell and spreads between cells utilizing actin-based motility (Gray et al. 2006). This pathogen has an ability to cross three human barriers: intestinal, blood-brain and fetoplacental. *L. monocytogenes* is a causative agent of listeriosis, which can be a fatal infection especially for elder people, immunocompromised individuals, and pregnant women (Alleberger and Wagner 2010). The fatality rate achieves 30%, so this disease represents a major public health concern. Listeriosis in neonates is one of three principal causes of bacterial meningitides. The infections of central nervous system are also described in adults with the mortality rate reaching even 60% (Vázquez-Bolland et al. 2001). *L. monocytogenes* produces several virulence factors; the major one is listeriolysin O (LLO), a pore-forming toxin belonging to the family of cholesterol-dependent cytolysins that is crucial for escape from vacuole after entry to the mammalian cell (Hamon et al. 2012). The activity of LLO is vital for inducing diarrhea and an inflammatory response after reaching intestinal tract (Barbuddhe and Chakraborty 2009).

The peptidoglycan (murein) constitutes the main compound of *L. monocytogenes* cell wall and plays a major role in *L. monocytogenes* pathogenesis (Boneca 2005). Its structure is unusual for Gram-positive bacteria, remaining this of Gram-negative species (e.g. *E. coli*) because of the presence of partially deacetylated *N*-acetyloglucosamine residues (Boneca et al. 2007). Another exceptional future of this pathogen is its ability to encode a high number of surface proteins what reflects the ability of *L. monocytogenes* to survive in a range of diverse environments (Bierne and Cossart 2007). *L. monocytogenes* is still susceptible to a variety of antibiotic but it should be mentioned that it is intrinsically resistant to a broad spectrum of cephalosporins commonly used in the therapy of many bacterial infections. The resistance to cephalosporins is based on several mechanisms including multidrug transporters and envelope proteins with a detoxification function (Krawczyk-Balska and Markiewicz 2016). In view of expanded resistance to antibiotics, the search for alternative therapies seems to be an urgent task.

The aim of the present study was to investigate the antibacterial effect of silver nanoparticles towards *L. monocytogenes* in order to identify the cellular target and mechanism of their activity.

Experimental

Materials and Methods

Bacterial strain, growth conditions and reagents used. Reference strain of *L. monocytogenes* PCM2191 was obtained from Polish Collection of Microorganisms (Institute of Immunology and Experimental Therapy in Wrocław, Poland). The strain was cultivated in tryptone soy yeast extract broth (TSYEB) medium (BTL, Poland) with constant shaking at 37°C. When required, the medium was supplemented with AgNPs and/or solidified with agar (15 g/l). Bacterial stock was stored in freezing solution containing 10% dimethyl sulfoxide (DMSO; v/v). All reagents were ultrapure and were purchased from Sigma, Germany.

Silver nanoparticles. Colloidal water solution of AgNPs was obtained from Nano-Tech (Warsaw, Poland). It contains nanosilver 4N in a concentration of 50 mg/kg, i.e. 50 ppm. The diameter of spherical nanoparticles varied from 2 nm to 35 nm, 70–75% of nanoparticles was within a range of 2–5 nm; their zeta potential was equal to 9.2 mV. Nanoparticles were synthesized by physical method according to the Polish Patent No. 3883399, starting from metallic silver (99.999%) and demineralized water. The detailed characteristics of the preparation used in all experiments was described previously (Chwalibóg et al. 2010).

Determination of MIC for AgNPs and their effect on *L. monocytogenes* growth and survival. For determination of minimal inhibitory concentration (MIC), the overnight culture of *L. monocytogenes* was diluted in fresh medium to the density of 2×10^6/ml colony forming units (cfu). The test was performed in 96-well polystyrene plates. To each well the equal volumes of 2-fold concentrated AgNPs suspension and bacterial inoculum were added and the plates were incubated at 37°C for 24 h in static condition. The MIC was determined within the concentration range of AgNPs from 0.5 g/ml to 12 μg/ml at 0.5 intervals. The sample without AgNPs constitutes an experimental control. The MIC value was considered as the lowest concentration entirely inhibiting bacterial growth, according to Clinical and Laboratory Standards Institute (CLSI) instruction. Three

independent repetitions were performed. To determine growth and survival curves, the overnight culture of *L. monocytogenes* was diluted in the fresh medium and incubated until the density of 10^7 cfu/ml was reached. Then the culture was divided into three equal volumes, AgNPs were added to two of them to a concentration of 0.25 MIC and 0.5 MIC, respectively. The third sample without AgNPs constituted experimental control. The samples were incubated for 24 h, the aliquots were taken for the first 5 h in 1-hour intervals, and finally after 24 h. Their absorbance (A_{600}) was read. Additionally, 0.1 ml aliquots appropriately diluted in saline were plated on solid media and after 24 h of incubation the colonies were counted.

Measurement of autolysis/lysis *of L. monocytogenes* cells. Overnight culture of *L. monocytogenes* was diluted in 50 ml of fresh medium to A_{600} equal to 0.1 and incubated until A_{600} equal to 0.6 was reached. Bacteria were centrifuged (10 min, 8000 × g) and the pellet, after washing twice in phosphate buffered saline (PBS), was resuspended in Tris-HCl buffer pH 8.0 containing Triton X-100 (0.1%) or lysozyme (20 μg/ml). Both suspensions were divided in three parts, one served as a control without AgNPs, to the reminding two the nanosilver suspensions in a concentration of 0.25 MIC and 0.5 MIC was added. After 60 min of incubation 0.1 ml aliquots appropriately diluted in saline were plated on solid media and after 24 h the colonies were counted.

Measurement *of L. monocytogenes* peptidoglycan autolysis rate. Overnight culture was divided in three parts, to two of them the suspension of AgNPs in a concentration of 0.25 MIC or 0.5 MIC were added, the third without AgNPs was left as a control. The procedure of peptidoglycan isolation and its absorbance measurement precisely followed those previously described (Kurek et al. 2010).

Estimation of AgNPs effect on permeability of *L. monocytogenes* envelopes. Overnight culture of *L. monocytogenes* was diluted to A_{600} equal to 0.1 and incubated further to A_{600} equal to 1.0. Then bacteria were spun down, the pellet was rinsed twice with 20 mM phoshate buffer, pH 7.1, and dissolved in this buffer to A_{600} equal to 0.8. A fluorescent dye, carboxyfluoresceine (cF), was added to the final concentration of 0.54 μM and after incubation at 40°C for 3 min three samples were prepared: the negative control without AgNPs and probes containing AgNPs at a concentration of 0.25 MIC or 0.5 MIC. The additional referential, positive control with 10% DSMO was also included. The samples were incubated at 37°C for 10 min, then the cells were spun down and the fluorescence of supernatants was measured in black 96-wells titration plates using fluorescence reader at wavelengths 490 nm (excitation) and 515 nm (emission), according to Johansen et al. 1997. To measure DNA and proteins release, the cell suspension prepared as described above was split into four parts: the negative control without AgNPs, samples containing AgNPs at a concentration of 0.25 MIC or 0.5 MIC, and positive control containing 100 μg/ml lysozyme. The samples were incubated at 37°C for 1 h, then cells were removed by centrifugation and the absorbance of supernatants at 260 nm (the released DNA) and 280 nm (the released proteins) was measured. This protocol was the modification of the procedure described previously (Markowska et al. 2014).

Statistical analysis The experiments were performed at least three times and every measurement was done in triplicate. The means ± standard deviations were calculated. Statistical significance of the difference between experimental samples was estimated using Student's test with Graphpad prism (ver. 6.0). p value < 0.05 was considered as statistically significant.

Results and Discussion

MIC of AgNPs was 8 μg/ml, and susceptibility of Gram-positive *L. monocytogenes* to AgNPs was higher than that observed for one of Gram-negative pathogens *Pseudomonas aeruginosa* ATCC 10145, for which MIC value was equal to 1 μg/ml (Markowska et al. 2014). The results presented in Fig. 1 showed that neither growth

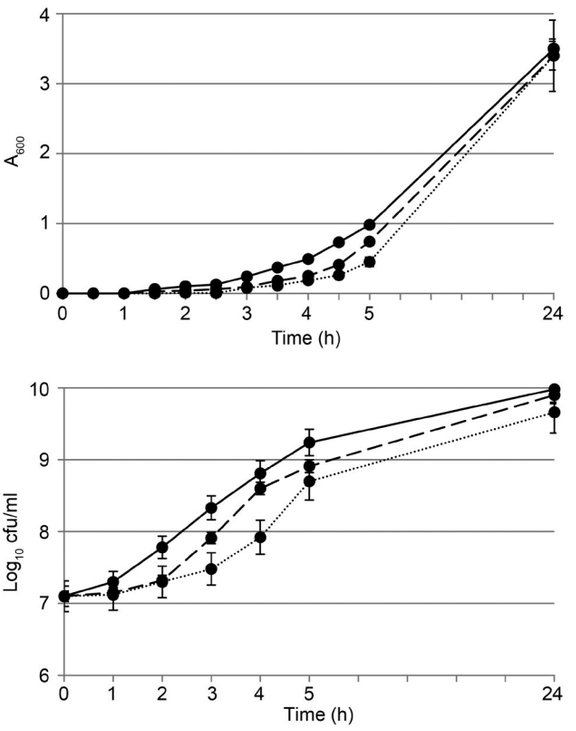

Fig. 1. Effect of AgNPs on *L. monocytogenes* growth (A) and survival (B).

Solid lines – control; dashed lines – 0.25 MIC AgNPs; dotted lines – 0.5 MIC AgNPs. Mean ± SD values of triplicate cultures were shown.

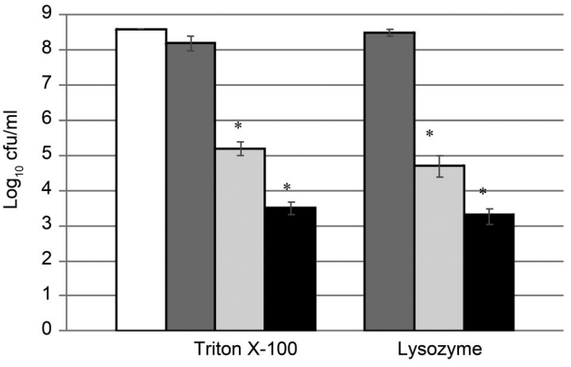

Fig. 2. Influence of AgNPs on lysozyme- or Triton X-100-induced autolysis/lysis of *L. monocytogenes*.

White bar – control, buffer only; dark grey bar – lysozyme or Triton X-100; light grey bar – lysozyme or Triton X-100 + 0.25 MIC AgNPs; black bar – lysozyme or Triton X-100 + 0.5 MIC AgNPs. The results are mean of three independent experiments with every measurement done in triplicate ± SD. Statistically relevant differences ($p < 0.05$) were marked with asterisks.

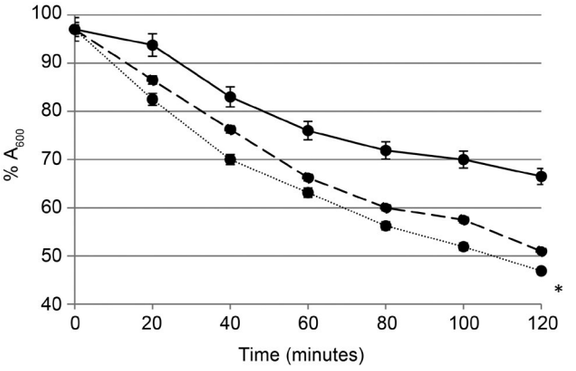

Fig. 3. Autolysis of isolated *L. monocytogenes* peptidoglycan in the presence of AgNPs.

Absorbance A_{600} at time 0 was considered as 100%. Solid line – control; dashed line – 0.25 MIC AgNPs; dotted line – 0.5 MIC AgNPs. The results are mean of three independent experiments with every measurement done in triplicate ± SD. Statistically relevant difference ($p < 0.05$) was marked with an asterisk.

nor survival of *L. monocytogenes* was substantially diminished in the presence of AgNPs in a concentration of 0.25 MIC or 0.5 MIC. In the subsequent experiments, AgNPs were used in these concentrations.

The effect of AgNPs on the autolysis/lysis of *L. monocytogenes* cells was studied in the cultures treated with nonionic surfactant Triton X-100 or lysozyme. The nonionic detergent Triton-X100 and lysosyme are the commonly used agents for induction of autolysis or lysis of *L. monocytogenes* cells, respectively (Smith et al. 1991; Popowska et al. 2009). When used in the moderate concentration, they can be applied to study the effect of various substances on their activity. The results of the experiments are presented in Fig. 2. In the control cultures, treated only with lysosyme or Triton X-100, 49% and 89% of cells survived, respectively. The observed killing effect of lysosyme was much weaker than that of Triton X-100 as it had already been presented by Kurek and coauthors (2010). The observed result can be due to the low level of glucosamine acetylation in *L. monocytogenes* peptidoglycan (Amano et al. 1977). The addition of AgNPs at a concentration of 0.25 MIC resulted in very high, over 1000-fold increase of the lysis of cultures treated with lysozyme or Triton X-100. AgNPs added in a concentration of 0.5 MIC caused further drop in the number of living cells.

The addition of nanosilver to peptidoglycan also caused the enhancement of peptidoglycan autolysis; however, this effect was not as pronounced as the effect observed for the whole cells. The drop in absorbance (A_{600}) of the control peptidoglycan sample was 33% after 2 h incubation in the buffer. In samples treated with AgNPs at the concentration of 0.25 MIC or 0.5 MIC the observed drop was 49% and 53%, respectively. Only the last value was statistically relevant, as it was shown in Fig. 3. It can be speculated that the AgNPs enhanced the ability of autolysins – peptidoglycan hydrolyzing enzymes which catalyse polymer destruction (Rice and Bayles 2008). Five *L. monocytogenes* autolysins have been identified (Popowska 2004); however, the analysis of the bacteria genome revealed the presence of more than twenty proteins with the putative peptidoglycan hydrolase domains (Bierne and Cossart 2007).

The effect of AgNPs on *L. monocytogenes* cells permeability was estimated by two methods. First, the efflux of cF dye was measured and after 10 min of treatment with AgNPs at a concentration of 0.5 MIC the efflux of cF was enhanced by 14% in comparison to the control sample, which was 79% relative to the total leakage determined after cell lysis with DMSO (Table I). In the second set of experiments the efflux of macromolecules, DNA and proteins, was studied by measuring respectively A_{260} and A_{280} of the supernatants of cultures treated with AgNPs at a concentration of 0.25 MIC or 0.5 MIC, in the control culture, and in the culture treated with lysozyme (positive control).

Table I
Increase in cF efflux from *L. monocytogenes* caused by AgNPs.

AgNPs concentration	% efflux of cF at time (min)	
	0	10
0 (negative control)	72 ± 2	79 ± 1
0.25 MIC	73 ± 2	84 ± 2
0.5 MIC	74 ± 2	93* ± 2

Efflux values are given as percentages relative to the total leakage determined after cell lysis with DSMO (means of three independent experiments with every measurement done in triplicate ± standard deviations are shown). Statistically relevant difference ($p < 0.05$) was marked with an asterisk.

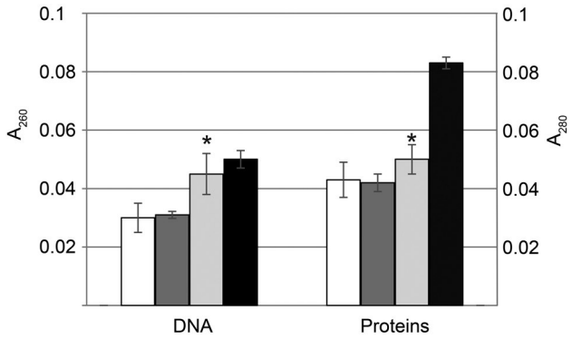

Fig. 4. Effect of AgNPs influence on the efflux of DNA and proteins from *L. monocytogenes*.

White bars – control, no AgNPs added; dark grey bars – 0.25 MIC AgNPs; light grey bars – 0.5 MIC AgNPs; black bars – 100 μg/ml lysozyme (positive control). The results are mean of three independent experiments with every measurement done in triplicate ± SD. Statistically relevant difference ($p < 0.05$) were marked with asterisks.

It was demonstrated that AgNPs in a concentration of 0.5 MIC enhanced DNA efflux by 48% after 60 min of treatment with AgNPs in comparison to control culture (Fig. 4). The efflux of proteins was enhanced by 30% after 60 min exposure to AgNPs at a concentration of 0.5 MIC. The maximal efflux of DNA and proteins caused by 60 min exposure to lysozyme amounted to 171% and 191% of the control sample without AgNPs. The observed enhancement of cF, DNA and proteins efflux as a result of AgNPs treatment points to the damage of cell wall. It was previously demonstrated that AgNPs are able to cover cells surface and to induce the formation of the hollows in cell envelopes what can results in the enhancement of cell permeability (Chwalibóg et al. 2010). In turn, AgNPs, when adsorbed on the cell surface, modify membranes potential what stimulates nanoparticles transport to the cytoplasm (Morones et al. 2005; Marambio-Jones and Hoek 2010).

Until now there have been only few papers describing the effect of silver nanoparticles on *L. monocytogenes* membranes. Microscopic analysis demonstrated deformation, disintegration and decrease in cell surface roughness of *L. monocytogenes* treated with silver nanoparticles synthesized by *Jatropa curcas* (Chauhan et al. 2016). It has also been demonstrated that AgNPs released from nanocomposites can penetrate the cell wall and plasma membrane of *L. monocytogenes* what results in separation of the cytoplasmic membrane from the cell wall (Tamayo et al. 2014). The results of our group demonstrated that AgNPs caused the decrease in *L. monocytogenes* cell length even by 50% what may also indicate their interaction with the cell wall (data not shown) (Milczarek 2015). It has been postulated recently that the activity of AgNPs against *L. monocytogenes* and the other foodborne pathogens make them useful in food industry, particularly in food packaging and food preservation (Patra and Baek 2017).

In conclusion, the original results presented here show that *L. monocytogenes* peptidoglycan is the target of AgNPs activity. This effect is demonstrated by the increase of cell autolysis and autolysis of the extracted peptidoglycan and also by the enhancement in cell permeability. Interference with *L. monocytogenes* cell wall integrity and functionality constitutes the important mechanism of nanosilver antibacterial activity towards this Gram-positive pathogen.

Acknowledgments

This research was supported by the Polish Ministry of Science and Higher Education intramural grant 140000/501/86-107429 through the Faculty of Biology, University of Warsaw. The authors are grateful to Nano-Tech for providing AgNPs. We also express many thanks to dr. Magdalena Popowska (Department of Applied Microbiology, Institute of Microbiology, University of Warsaw) for critical reading of the manuscript.

Literature

Alleberger F, Wagner M. 2010. Listeriosis: a resurgent foodborne infection. Clin Microbiol Infect. 16:16–23.

Amano K, Hayashi H, Araki Y, Ito E. 1977. The action of lysozyme on peptidoglycan with N-unsubstituted glucosamine residues. Isolation of glycan fragments and their susceptibility to lysozyme. Eur J Biochem. 76:299–307.

Barbuddhe SB, Chakraborty T. 2009. *Listeria* as an enteroinvasive gastrointestinal pathogen. Curr Top Microbiol Immunol. 337: 173–195.

Bierne H, Cossart P. 2007. *Listeria monocytogenes* surface proteins: from genome predictions to function. Microbiol Mol Biol Rev. 71:377–397.

Birla SS, Tiwari VV, Gade AK, Ingle AP, Yadav AP, Rai MK. 2009. Fabrication of silver nanoparticles by *Phoma glomerata* and its combined effect against *Escheriochia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aures*. Lett Appl Microbiol. 48:173–179.

Boneca IG. 2005. The role of peptidoglycan in pathogenesis. Curr Opin Microbiol. 8:46–53.

Boneca IG, Dussurget O, Cabanes D, Nahori MA, Sousa S, Lecuit M, Psylinakis E, Bouriotis V, Hugot JP, Giovannini M, et al. 2007. A critical role for peptodoglycan *N*-deacetylation in *Listeria* evasion from the host innate immune system. Proc Natl Acad Sci USA. 104:997–1002.

Chauhan N, Tyaghi AK, Kumar P, Malik A. 2016. Antibacterial potential of *Jatropha curcas* synthesized silver nanoparticles against food borne pathogens. Front Microbiol. 7:1748.

Chwalibóg A, Sawosz E, Hotowy A, Szeliga J, Mitura S, Mitura K, Grodzik M, Orlowski P, Sokolowska A. 2010. Visualization of interaction between inorganic nanoparticles and bacteria or fungi. Int J Nanomed. 5:1085–1094.

Gray MJ, Freitag NE, Boor KJ. 2006. How the bacterial pathogen *Listeria monocytogenes* mediates the switch from environmental Dr. Jekyll to pathogenic Mr. Hyde. Infect Immun. 74:2505–2512.

Hamon MA, Ribet D, Stavru F, Cossart P. 2012. Listeriolysin O: the Swiss army knife of *Listeria*. Trends Microbiol. 20:360–368.

Ingle A, Gade A, Pierrat S, Sonnichsen C, Rai M. 2008. Mycosynthesis of silver nanoparticles using the fungus *Fusarium*

acuminatum and its activity against some human pathogenic bacteria. Curr Nanotechnol. 4:141–144.

Johansen C, Verheul A, Gram L, T Gill, Abee T. 1997. Protamine-induced permeabilization of cell envelopes of Gram-positive and Gram-negative bacteria. Appl Environ Microbiol. 63:1155–1159.

Krawczyk-Balska A, Markiewicz Z. 2016. The intrinsic cephalosporin resistome of *Listeria monocytogenes* in the context of stress response, gene regulation, pathogenesis and therapeutics. J Appl Microbiol. 120:251–265.

Kurek A, Grudniak AM, Szwed M, Klicka A, Samluk Ł, Wolska KI, Janiszowska W, Popowska M. 2010. Oleanolic acid and ursolic acid affect peptidoglycan metabolism in *Listeria monocytogenes*. Antonie van Leeuwenhoek Int J Gen Mol Microbiol. 97:61–68.

Marambio-Jones C, Hoek EMV. 2010. A review of the antibacterial effects of silver nanomaterials and potential implications. J Nanoparticle Res. 12:1531–1551.

Markowska K, Grudniak AM, Wolska KI. 2013. Silver nanoparticles as an alternative strategy against bacterial biofilms. Acta Biochim Pol. 60:523–530.

Markowska K, Grudniak AM, Krawczyk K, Wróbel I, Wolska KI. 2014. Modulation of antibiotic resistance and induction of stress response in *Pseudomonas aeruginosa* by silver nanoparticles. J Med Microbiol. 63:849–854.

Milczarek BE. 2015. Influence of silver nanoparticles on *Listeria monocytogenes* cell membranes. Ph. D. Thesis. Warsaw (Poland): University of Warsaw.

Morones JR, Elechigerra JL, Camacho A, Ramirez JT. 2005. The bactericidal effect of silver nanoparticles. Nanotechnology. 16: 2346–2353.

Patra JK, Baek KH. 2017. Antibacterial activity and synergistic antibacterial potential of biosynthesized silver nanoparticles against foodborne pathogenic bacteria along with its anticandidal and antioxidant effect. Front Microbiol. 8:167.

Popowska M. 2004. Analysis of the peptidoglycan hydrolases of *Listeria monocytogenes*: multiple enzymes with multiple functions. Pol J Microbiol. 53:29–34.

Popowska M, Kusio M, Szymańska P, Markiewicz Z. 2009. Inactivation of the wall-associated de-N-acetylase (PgdA) of *Listeria monocytogenes* results in greater susceptibility of the cells to induced autolysis. J Microbiol Biotechnol. 19:932–945.

Rai M, Yadav A, Gade A. 2009. Silver nanoparticles as a new generation of antimicrobials. Biotechnol Adv. 27:76–83.

Rice KC, Bayles KW. 2008. Molecular control of bacterial death and lysis. Microbiol Mol Biol Bev. 72:85–109.

Shahverdi AR, Fakhimi A, Shahverdi HR, Minaian SM. 2007. Synthesis and effect of silver nanoparticles on the antibacterial activity against *Staphylococcus aureus* and *Escherichia coli*. Nanomedicine 3:168–171.

Singh M, Singh S, Prasada S, Gambhir IS. 2008. Nanotechnology in medicine and antibacterial effect of silver nanoparticles. Digest J Nanomat Biostruct. 3:115–122.

Smith JL, McColgan C, Marmer BS. 1991. Growth temperature and the action of lysozyme on *Listeria monocytogenes*. J Food Sci. 56:1101–1102.

Stapsford KE, Tyner KM, Dair BJ, Deschamps JR, Medintz IL. 2011. Analyzing nanomaterial bioconjugates: a review of current and emerging purification and characterization techniques. Anal Chem. 83:4453–4488.

Tamayo LA, Zapata PA, Vejar ND, Azócar MI, Gulppi MA, Zhou X, Thompson GE, Rabagliati FM, Páez MA. 2014 Release of silver and copper nanoparticles from polyethylene nanocomposites and their penetration into *Listeria monocytogenes*. Mater Sci Eng C Mater Biol Appl. 40:24–31.

Vázquez-Bolland JA, Kuhn M, Berche P, Chakraborthy T, Dominguez-Bernal G, Goebel W, González-Zorn B, Wehland J, Kreft J. 2001. *Listeria* pathogenesis and molecular virulence determinants. Clin Microbiol Rev. 14:584–640.

Wolska KI, Grudniak AM, Kamiński K, Markowska K. 2015. The potential of metal nanoparticles for inhibition of bacterial biofilms. In: Rai M, Kon K, editors. Nanotechnology in Diagnosis, Treatment and Prophylaxis of Infectious Diseases. Amsterdam (Netherlands): AP Elsevier. p. 119–132.

Polish Journal of Microbiology
2018, Vol. 67, No 3, 321–331
https://doi.org/10.21307/pjm-2018-039

ORIGINAL PAPER

Isolation of Bacterial Endophytes from *Phalaris arundinacea* and their Potential in Diclofenac and Sulfamethoxazole Degradation

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Submitted 30 October 2017, revised 21 February 2018, accepted 20 May 2018

Abstract

Diclofenac (DCF), a non-steroidal anti-inflammatory drug (NSAID) and sulfamethoxazole (SMX), an antimicrobial agent, are in common use and can be often detected in the environment. The constructed wetland systems (CWs) are one of the technologies to remove them from the aquatic environment. The final effect of the treatment processes depends on many factors, including the interaction between plants and the plant-associated microorganisms present in the system. Bacteria living inside the plant as endophytes are exposed to secondary metabolites in the tissues. Therefore, they can possess the potential to degrade aromatic structures, including residues of pharmaceuticals. The endophytic strain MG7 identified as *Microbacterium* sp., obtained from root tissues of *Phalaris arundinacea* exposed to DCF and SMX was tested for the ability to remove 2 mg/l of SMX and DCF in monosubstrate cultures and in the presence of phenol as an additional carbon source. The MG7 strain was able to remove approximately 15% of DCF and 9% of SMX after 20 days of monosubstrate culture. However, a decrease in the optical density of the MG7 strain cultures was observed, caused by an insufficient carbon source for bacterial growth and proliferation. The adsorption of pharmaceuticals onto autoclaved cells was negligible, which confirmed that the tested strain was directly involved in the removal of DCF and SMX. In the presence of phenol as the additional carbon source, the MG7 strain was able to remove approximately 35% of DCF and 61% of SMX, while an increase in the optical density of the cultures was noted. The higher removal efficiency can be explained by adaptive mechanisms in microorganisms exposed to phenol (i.e. changes in the composition of membrane lipids) and by a co-metabolic mechanism, where non-growth substrates can be transformed by non-specific enzymes. The presence of both DCF and SMX and the influence of the supply frequency of CWs with the contaminated wastewater on the diversity of whole endophytic bacterial communities were demonstrated. The results of this study suggest the capability of the MG7 strain to degrade DCF and SMX. This finding deserves further investigations to improve wastewater treatment in CWs with the possible use of pharmaceuticals-degrading endophytes.

Key words: endophytic bacteria, constructed wetlands, diclofenac, sulfamethoxazole, biodegradation

Introduction

The presence of pharmaceutical products in the environment has become a subject of great interest due to their widespread use and disposal. Belonging to this group, diclofenac (DCF, [2-(2,6-dichloro anilino)phenyl]acetic acid) and sulfamethoxazole (SMX, 4-amino-N-(5-methylisoxazol-3-yl)-benzenesulfonamide) are in common use.

Diclofenac is a non-steroidal anti-inflammatory drug (NSAID) administered to reduce inflammation and for relieving pain in patients. Recent studies have estimated that, at present, on average of 1443 ± 58 tons of DCF are consumed globally (Acuña et al. 2015). DCF sold OTC (over-the-counter) may also hamper any efforts to quantify its use. Due to DCF's high resistance to biodegradation and harmful impact on some environmental species in low concentration (≤ 1 µg/l) (Schwaiger et al. 2004), in 2015 the drug was included in the first Watch List of substances in the EU that require environmental monitoring in all Member States (Barbosa et al. 2016).

Sulfonamides, including sulfamethoxazole (SMX), belong to the most commonly used antibiotics in clinical (in combination with trimethoprim) and veterinary medicine (Sukul and Spiteller 2006), and are applied to treat bacterial and protozoal infections. The worldwide consumption of antibiotics ranges from 100 000 to 200 000 tons per year and more than 20 000 tons of sulfonamides could be introduced to

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the environment each year (Baran et al. 2011). Antibiotics affect the environmental microbiome and pose the problems of development and the propagation of antibiotic resistance (Gao et al. 2012).

Both DCF and SMX are known to be recalcitrant to degradation in conventional activated sludge treatment systems and have been detected in wastewater effluents (Vieno and Sillanpää 2014), surface water (Hirsch et al. 1999), ground water (Sui et al. 2015) and even drinking water (Schaider et al. 2013). Therefore, searching for an alternative, and at the same time, effective methods of their removal, is still a challenge for researchers. Verlicchi and Zambello (2014) suggested that constructed wetlands (CWs) can represent a promising approach for treating wastewater containing recalcitrant compounds. CWs are engineered systems that use plants and plant-associated microorganisms' ability to remove contaminants from wastewater (Shelef et al. 2013). The effectiveness of these systems in wastewater treatment depends, e.g., on the presence and activity of plant-associated microorganisms, especially endophytic bacteria. They live inside plant tissues without causing any apparent symptoms of disease and can play a crucial role in the biodegradation or biotransformation of organic pollutants (Weyens et al. 2009). Recent studies have described many species of endophytes that are able to remove or transform different xenobiotics (Afzal et al. 2014). However, only few of these studies have focused on the degradation of pharmaceuticals (Sauvêtre and Schröder 2015; Syranidou et al. 2016).

Despite the ubiquity of pharmaceuticals, the response of the whole endophytic bacterial communities to the pressure caused by the presence of DCF and SMX, as well as the abilities of bacterial endophytes to degrade or transform these pharmaceuticals, are still not completely understood. Thus, the objectives of this study were: (i) the isolation of endophytic bacteria from the root tissues of *Phalaris arundinacea* (reed canary grass), taken from lab-scale CWs in which the plants were exposed or non-exposed to DCF and SMX pollution; (ii) the determination of the degradation potential of the selected endophytic isolate using DCF and SMX as the sole carbon source and in the presence of phenol as an additional carbon source in batch liquid cultures; (iii) the evaluation of diversity of endophytic bacterial communities in root tissues using PCR-DGGE.

Experimental

Materials and Methods

Plant source. The plant samples were obtained from a lab-scale constructed wetland system, consisting of 12 columns simulating the operation of intermittent downflow beds (Fig. 1). The columns were planted with reed canary grass. The CW system was fed with synthetic municipal wastewater (Nopens et al. 2001), which consisted of (mg/l): 208.76 Urea, 264 Yeast Extract, 118 Skim Milk Powder, 510.4 CH_3COONa, 40 Peptone, 41.37 KH_2PO_4, 0.62 NH_4Cl, 0.96 $KCr(SO_4)_2 \times 12H_2O$, 0.781 $CuSO_4 \times 5H_2O$, 0.108 $MnSO_4 \times H_2O$, 0.359 $NiSO_4 \times 7H_2O$, 0.1 $PbCl_2$, 0.208 $ZnCl_2$, 4.408 $MgSO_4 \times 7H_2O$, 11.6 $FeSO_4 \times 7H_2O$. Six columns (A1-3 and B1-3) were fed 4 times per day with a 0.35 l of wastewater, while the rest of columns (C1-3 and D1-3) were fed once a day with 1.4 l of wastewater.

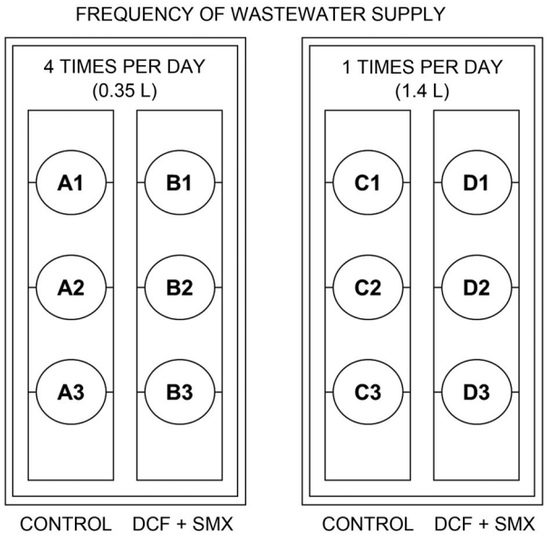

Fig. 1. The scheme of a lab-scale constructed wetlands system, from which the plant samples were collected.

The experimental system was operated using a high-pressure sodium lighting system with light/dark conditions that changed according to the season: for fall, 9 h of light and 15 h of darkness (9/15) and, for winter, 8/16. The mean night and day temperature during the experiment was 23.1°C and 29.1°C, and the mean humidity was 59.3%. DCF and SMX were added to the synthetic wastewater to a final concentration of 2 mg/l and were dosed to columns B1-3 and D1-3. A detailed description of the technical part of the experiment is provided in previous publication (Nowrotek et al. 2016).

Isolation of endophytic bacteria. The roots obtained from the columns not exposed and exposed to DCF and SMX mixed pollution, were separately sliced into 1–2 cm pieces, weighed and their surface was sterilized with 2% NaClO solution for 10 min. Afterwards, the samples were rinsed three times in sterile water for 1 min and dried on sterilized filter paper. Then, the samples were homogenized in a sterile ceramic mortar in 5 ml of phosphate-buffered saline (PBS). The crushed root tissues were used to obtain tenfold serial dilutions (10^{-1}, 10^{-2}, 10^{-3}) and 100 µl of each dilution was spread

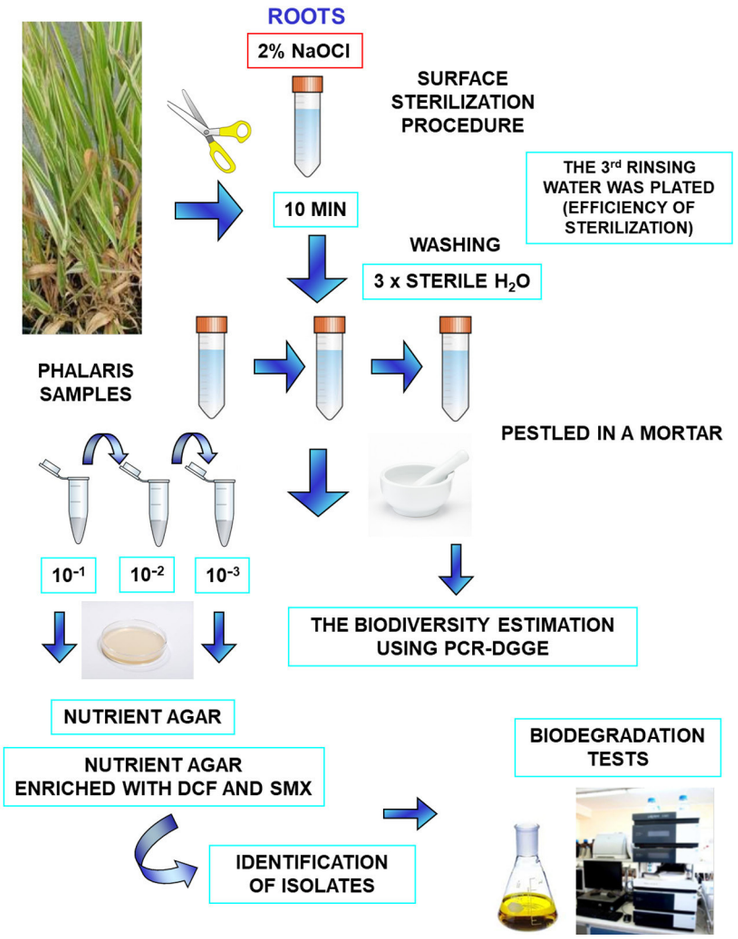

Fig. 2. Workflow for isolation of cultivable endophytic bacteria from *P. arundinacea* plants non-exposed and exposed to DCF and SMX mixed pollution (three plants per condition from each column were harvested).

onto nutrient agar plates enriched with DCF and SMX (2 mg/l, both). All plates were prepared in triplicate and incubated at 25°C for 7 days. A 100 µl sample of the third rinsing water was plated to confirm the efficiency of sterilization. The colonies on the plates were counted and the number of colony forming units per gram of fresh weight (CFU/g FW) was determined. The averages and standard errors were calculated from six replicates from a mixed sample of plants from three columns. The average CFU/g FW obtained after counting of the colonies grown on nutrient agar and nutrient agar enriched with DCF and SMX were compared using an unpaired two-sided t-test. The workflow for isolation of cultivable endophytic bacteria from reed canary grass is shown in Fig. 2.

Identification of selected endophytic bacteria. The isolates, representing the most abundant colony types grown on medium enriched with pharmaceuticals, were selected and identified. Genomic DNA was extracted from pure culture using the FastDNA™ SPIN kit for soil (MP Biomedicals) according to the recommendations of the manufacturer. The primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1401R (5'-CGGTGTGTACAAGACCC-3') were used to partially amplify the 16S rRNA gene. The reaction mixtures contained: 1 × buffer, 2 mM $MgCl_2$, 5 pM/µl of 27F and 1492R primers, 20 pM/µl dNTPs and 1.5 U GoTAQ Flexi (Promega) in total reaction volume of 30 µl. Extracted DNA at a concentration of 0.15–0.2 µg/µl was added to the PCR mixture. After initial denaturation at 94°C for 5 min, each thermal cycling involved: 30 s denaturation at 95°C, 45 s annealing at 57°C and 1.5 min elongation at 72°C. After 30 cycles, a final elongation was performed at 72°C for 7 min. The presence of amplicons was confirmed by gel electrophoresis on 1% agarose (w/v) according to standard procedure. Using Clean Up Kit (A&A Biotechnology), PCR products were purified. Then, they were reamplified and sequenced with the BigDye®Terminator v3.1 kit (Applied Biosystems). The sequence of DNA was compared with GenBank NCBI (National Center for Biotechnology Information).

Biodegradation of DCF and SMX. For the biodegradation study, the isolate (MG7) representing the most abundant colony types grown on the medium enriched with DCF and SMX, was selected. The strain was cultivated in a nutrient broth at 30°C with agitation at 130 rpm for 24 h. Bacterial cells were harvested by centrifugation (5000 × g at 4°C for 15 min), washed with a sterile mineral salt medium, and used as an inoculum in experiments on the degradation of DCF and SMX.

The biological degradation of DCF and SMX in monosubstrate and in the presence of phenol as the additional carbon source was carried in 250-ml Erlenmeyer flasks containing 100 ml of a sterile mineral salt medium, which consisted of (g/l): 3.78 $Na_2HPO_4 \times$ $\times 12H_2O$, 0.5 KH_2PO_4, 5.0 NH_4Cl, 0.2 $MgSO_4 \times 7H_2O$ and 0.01 yeast extract. The inoculum of bacterial cells prepared as described above was added to the medium to an initial optical density of about 0.2 to 0.4 at $\lambda = 600$ nm. Additionally, two control cultures (100 ml) were prepared: an uninoculated control involved the mineral salt medium and pharmaceuticals (abiotic control), and a heat-killed control consisted of bacterial cells destroyed by autoclaving (adsorption control). The optical density of the heat-killed control was the same as for the tested cultures.

DCF and SMX were added to each flask to a final concentration of 2 mg/l. In the monosubstrate culture, DCF or SMX was the sole carbon and energy source. In the flasks enriched with phenol as the additional carbon source, 20 mg/l of phenol was added every 5 days of the culture, to give a final concentration of 100 mg/l. All cultures were grown in triplicate and incubated with shaking at 130 rpm at 25°C for 20 days.

Determination of DCF and SMX concentration. High-performance liquid chromatography, coupled with variable wavelength detector (UltiMate 3000 system; Dionex Corporation, Sunnyvale, CA, USA), was used for quantification of SMX and DCF concentrations in all cultures. The chromatographic separation was performed using C18 Hypersil TM Gold column (250 mm × 4.6 mm; pore size: 5 µm) (Thermo Scientific, Polygen, Poland). The mobile phase consisted of a mixture of acetonitrile and acetate buffer (pH = 5.7), in a volumetric ratio 40:60 (v/v). During the analysis, an isocratic flow rate was used, and it was set at 1.0 ml/min. In the above-described conditions, the retention times (RT) of the compounds investigated were equal to 8.4–0.3 min and 6.4–0.2 min for DCF and SMX, respectively. The limit of quantification of DCF and SMX was equal to 0.2 mg/l. It was established as the first lowest calibration point of their calibration curves (linear regression, $R2 > 0.999$). The calculated value of "signal to noise" ratio in the case of both compounds investigated was greater than 10. The method was validated according to ISO/IEC 17025 (2005), which means that all method parameters as: accuracy, precision and recovery meet the criteria of this ISO standard. The analyses were performed and confirmed at four different wavelengths, i.e., 220 nm; 240 nm; 268 nm and 280 nm. The data was evaluated by means of Dionex Chromeleon™ 6.8 software.

The efficiency of removal was calculated using the formula:

$$\%R = (Cas - Cs)/Cac \times 100\%$$

where Cs is concentration of DCF or SMX in the sample, Cas is the concentration of DCF or SMX in the appropriate abiotic control.

Biodiversity of endophytic bacteria. All genomic DNA from tissues homogenates, obtained from surface sterilized roots of *P. arundinacea*, was extracted using FastDNA® SPIN KIT FOR SOIL (MP Biomedicals, USA), according to manufacturer's instructions and stored at –20°C until PCR amplification. Primers (Muyzer et al. 1993): 338f (5' CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG 3') and 518r (5' ATT ACC GCG GCT GCT GG 3') were used for partial 16S rRNA bacterial gene PCR amplification. PCR was carried out in a 30 µl (total volume) reaction mixture containing: 19 µl sterile MiliQ water, 6 µl PCR buffer (GoFlexi TAQ, Promega), 2.4 µl $MgCl_2$ (2 mM), 0.25 µl of both primers (5 pmol/µl), 1.3 µl dNTPs (20 pmol/µl), 0.5 µl of genomic DNA (0,15–0,2 µg/µl) and 0.3 µl Taq DNA polymerase (1.5 U).

PCR amplification was performed using an Eppendorf thermal cycler and the following steps: (i) the initial denaturation step (10 min at 95°C); (ii) 30 cycles, each single cycle consisting of denaturation (10 min at 95°C), annealing (1 min at 53°C), and elongation (2 min at 72°C); and (iii) the final extension step (12 min at 72°C). Products were analyzed in agarose gel (0.8% w/vol agarose, 1 × TBE buffer), stained with ethidium bromide (1% w/vol) in MiliQ water and photographed under UV light. The DGGE of PCR products obtained in reaction with 338f and 518r primers were performed using the Dcode Universal Mutation Detection System (BioRad). The polyacrylamide gel (8% (v/v) with a gradient of 30–60% denaturant) was run for 9 h at 55 V in a 1 × TAE buffer at a constant temperature of 60°C. The gel was stained with SYBR GOLD (1:10 000, Invitrogen) in MiliQ water for 20 min and washed in MiliQ water twice for 15 min, then visualized under UV light and photographed. The DGGE banding patterns with 16S rDNA PCR products were analyzed using Quantity One 1D Software (BioRad). The structural diversity of the bacterial communities was calculated based on the Shannon-Weaver diversity index, H' (Shannon and Weaver 1963), estimated from the relative band intensities obtained from the DGGE fingerprints.

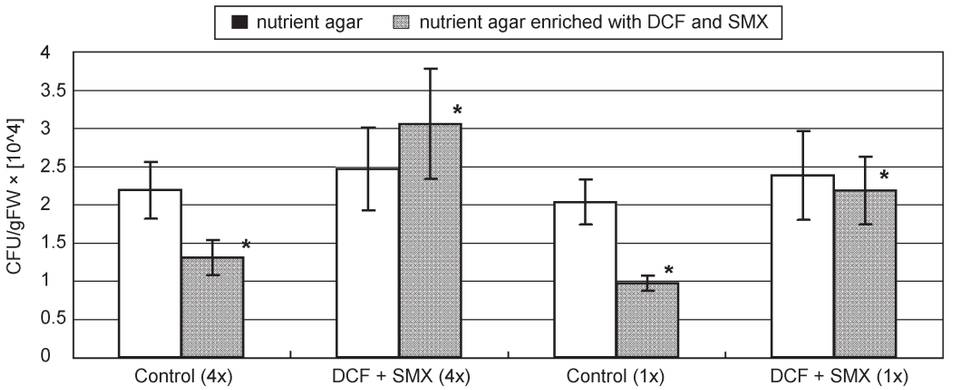

Fig. 3. The number of bacteria (CFU/g FW) obtained from the surface of the sterilized reed canary grass root samples. Light bars – the number of bacterial endophytes grown on nutrient agar. Grey bars – the number of bacterial endophytes grown on nutrient medium enriched with DCF and SMX. N = 6. Significant differences have been marked using * ($p < 0.05$; t-test). Time regime of wastewater supply in brackets.

Results

Characterization of endophytic bacteria. Endophytic bacteria were found in *P. arundinacea* root tissues not exposed and exposed to DCF and SMX. After seven days of incubation, no significant differences in CFU/g fresh weight (FW) were observed, when nutrient agar was used as the growth medium (Fig. 3). The number of endophytic bacteria was comparable for the samples not exposed and exposed to DCF and SMX mixed pollution. When nutrient agar was enriched with DCF and SMX and used as the growth medium, significant differences in CFU/g FW were noticed. The number of endophytic bacteria was higher in the samples exposed to DCF and SMX mixture. However, the diversity of colony morphologies was lower than in the samples non-exposed to pharmaceuticals. The time regime of wastewater supply to CWs did not affect the CFU/g FW values.

Twelve isolates showing different colony morphologies were selected from the medium enriched with pharmaceuticals and transferred to a new plate. Cultures were considered to be pure after two successive passages on streak plates. Four isolates, representing the most abundant colony types grown on the medium enriched with pharmaceuticals, were identified by using partial 16S rRNA gene sequencing. The results are shown in Table I.

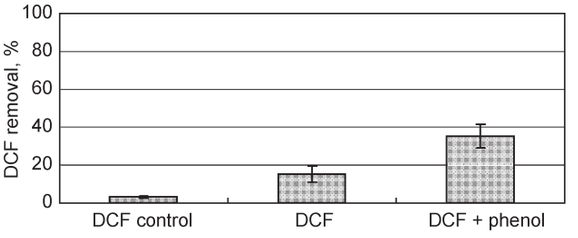

Fig. 4. The removal of 2 mg/l DCF by MG7 strain in the adsorption control, without additional carbon source and with phenol at a concentration of 100 mg/l as the additional carbon source.

Biodegradation of DCF and SMX. For the biodegradation test, the isolate (MG7) representing the most abundant colony types grown on the medium enriched with DCF and SMX was selected. In the first set of experiments, DCF and SMX were used as a single substrate at a concentration of 2 mg/l, respectively. After 20 days of incubation, strain MG7 removed approximately 15.23 ± 4.21% of DCF (Fig. 4) and 9.12 ± 3.56% of SMX (Fig. 5). A decrease in the optical density of the cultures was observed, from 0.350 to 0.200 (Fig. 6) and from 0.387 to 0.198 (Fig. 7), respectively. The removal of DCF and SMX in the adsorption cultures was low, and was equal to 3.04 ± 0.56% and 2.41 ± 1.01%, respectively.

In the cultures enriched with phenol, as an additional carbon source, an improvement of SMX removal efficiency was observed, and 61.24 ± 5.09% of this

Table I
Identification of bacterial endophytes, based on the 16S rRNA gene sequence.

Isolate	Clostest match	Similarity, %	Sequence ID
MG2	*Variovorax boronicumulans*	96	NR_114214.1
MG5	*Bacillus wiedmannii*	97	NR_152692.1
MG7	*Microbacterium flavescens*	98	NR_029350.1
MG11	*Agrobacterium tumefaciens*	97	NR_041396.1

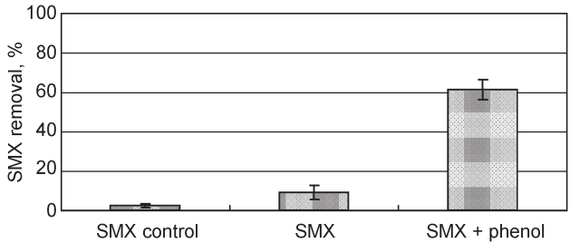

Fig. 5. The removal of 2 mg/l SMX by MG7 strain in the adsorption control, without additional carbon source and with phenol at a concentration of 100 mg/l as the additional carbon source.

substrate was removed after 20 days of incubation (Fig. 5). Moreover, an increase in the optical density of MG7 strain was observed (from 0.256 to 0.721) (Fig. 7). The efficiency of DCF removal was lower (35.23 ± 6.17%) (Fig. 4) in comparison to the cultures with SMX, as well as an increase in the optical density was not as efficient as it was observed for the SMX-containing cultures (from 0.321 to 0.509) (Fig. 6).

Biodiversity of endophytic bacteria. Total DNA extracts, obtained from the surface-sterilized root tissues of *P. arundinacea* non-exposed and exposed to DCF and SMX, were subjected to PCR amplification, followed by analysis *via* DGGE. The fingerprints obtained from DGGE separation of 16S rRNA gene fragments are shown in Fig. 8. All the samples yielded similar DGGE profiles. Some bands (signed as 3, 7 and 8) appeared in all samples. Bands signed as 6 were visible only in samples obtained from columns B1-3 and D1-3, in which plants were exposed to DCF and SMX. Bands signed as 5 appeared in all samples, except the samples obtained from columns B1-3. Bands signed as 4 were visible only in samples obtained from columns A1-3 and B1-3, i.e. columns exposed to wastewater four times a day. Bands signed as 1 and 9 appeared in samples obtained from columns D1-3 and C1-3, respectively. In profiles obtained from columns D1-3, there was a lack of the band signed as 2, visible in the other samples.

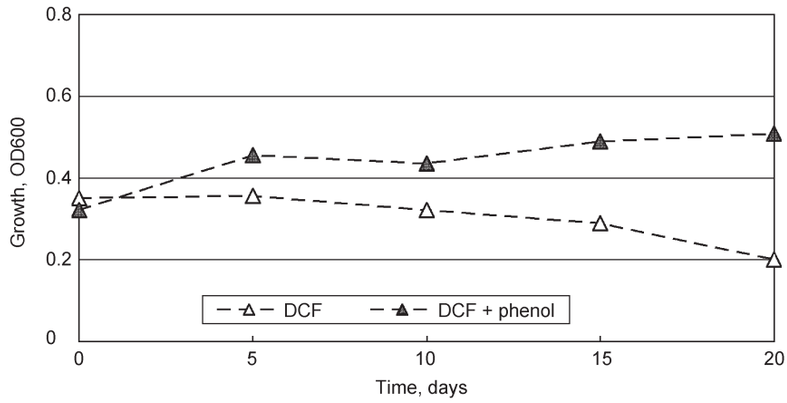

Fig. 6. Changes in biomass of MG7 strain in the cultures that contained DCF, monitored as optical density at 600 nm, without additional carbon source and with phenol at a concentration of 100 mg/l as additional carbon source.

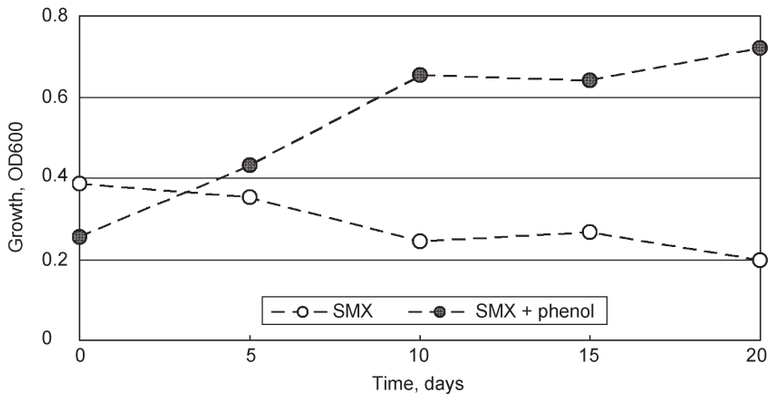

Fig. 7. Changes in biomass of MG7 strain in the cultures that contained SMX, monitored as optical density at 600 nm, without additional carbon source and with phenol at a concentration of 100 mg/l as additional carbon source.

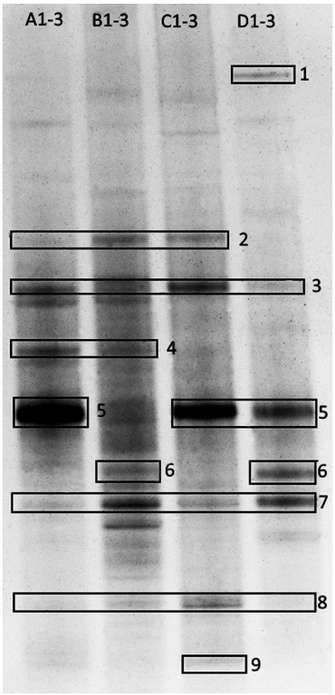

Fig. 8. DGGE pattern of 16S rRNA gene fragments of 180 bp amplified from DNA obtained from the surface-sterilized root tissues of *P. arundinacea*.

A1-3, C1-3 – the root samples non-exposed to DCF and SMX, B1-3, D1-3 – the root samples exposed to DCF and SMX. The frequency of wastewater supply: A1-3 and B1-3 – 0.35 l four times a day; C1-3 and D1-3 1.4 l – once a day. Description of the bands in the manuscript text.

The Shannon biodiversity index was determined based on densitometry analysis. The results are presented in Fig. 9. The highest value of Shannon biodiversity index was calculated for the sample obtained from columns B1-3, in which plants were exposed to DCF and SMX and the lowest for the sample obtained from the columns C1-3, which were non-exposed to DCF and SMX. The values of Shannon biodiversity index were comparable for all samples.

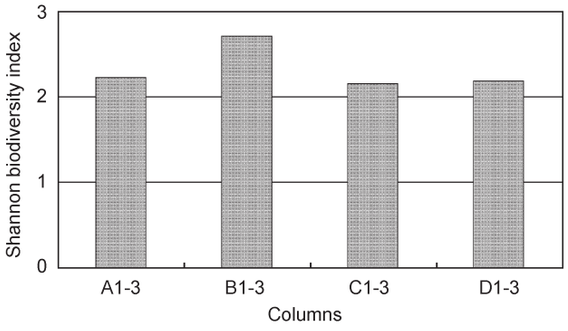

Fig. 9. Shannon biodiversity index based on DGGE profiles.

A1-3, C1-3 – the root samples non-exposed on DCF and SMX; B1-3, D1-3 – the root samples exposed on DCF and SMX. The frequency of wastewater supply: A1-3 and B1-3 – 0.35 l four times a day; C1-3 and D1-3 – 1.4 l once a day.

Discussion

Biological methods have been increasingly used for the removal of various xenobiotics, including pharmaceuticals from the environment. Constructed wetlands could be a sustainable solution for wastewater treatment in small villages and developing countries (Zhang et al. 2014), for a final treatment of troublesome effluents (Hijosa-Valsero et al. 2016) or the wastewater containing selected anthropogenic (micro)pollutants (Felis et al. 2016). Microbial endophytes reside within plant tissues and are therefore exposed to many plant metabolites (including phenolic compounds) and could possess efficient enzymatic systems for their transformation. Siciliano et al. (2001) showed that plants grown in soil contaminated with xenobiotics naturally recruited endophytes with the necessary contaminant-degrading capabilities. Inside the plant, the first step of different xenobiotic removal could be supported by the endophytic communities living in roots and rhizomes. Therefore, biodegradation of such compounds using plant-associated endophytic bacteria, has recently gained considerable attention.

In our investigations, the number of endophytic bacteria from roots non-exposed and exposed to DCF and SMX was determined. It was expected that pharmaceuticals, in particular SMX, would exert a selective pressure on endobacterial communities. The number of endophytic bacteria grown on nutrient medium were comparable and the decrease of this value in the samples exposed to DCF and SMX was not observed. However, the diversity of the colony morphologies was lower. When the nutrient agar enriched with DCF and SMX was used as the growth medium, significant differences in CFU/g fresh weight (FW) were observed. A higher number of endophytic bacteria was noticed in the samples exposed to DCF and SMX, what suggests that isolates can be better adapted to the presence of pharmaceuticals and be able to use these compounds as a carbon source or they can be resistant to SMX. A higher number of endophytic bacteria was observed in the samples obtained from *C. pepo* exposed to DDE (degradation product of DDT) (Evers et al. 2016) and the authors concluded that the xenobiotic can be used as a growth substrate by endophytes.

For biodegradation tests, the isolate (MG7) representing the most abundant colony types grown on medium enriched with DCF and SMX, identified by partial 16S rRNA gene sequencing as *Microbacterium flavescens,* was selected. The general potential of different *Microbacterium* species for the biodegradation of pharmaceuticals has already been highlighted (Kim et al. 2011; Ricken et al. 2013; Ricken et al. 2017).

In our investigations, the MG7 strain removed approximately 9% of SMX and 15% of DCF (the initial

concentration was 2 mg/l, each) from the liquid medium in monosubstrate culture. However, a decrease in the optical density of the cultures was also observed. One reason could be that the applied carbon and energy source didn't provide enough energy for bacterial growth and proliferation. When the carbon source was used up, the dying cells could lyse and provide a source of nutrients (Maier 2000). However, this hypothesis should be confirmed in follow-up studies focusing on endogenous metabolism. The literature data shows that the decrease in the optical density can be used for determination of bacterial cell lysis (Yin et al. 2013; Danevčič et al. 2016). A decrease in the optical density can be observed because of cellular damage and the leakage of cytoplasmic contents. The refractive index of bacterial suspension decreases and therefore, a decline in optical density can be noted because of lower light scattering.

The experiments on the autoclaved bacterial cells (heat-killed) showed that the removal of DCF and SMX was low and equal to 3.04 ± 0.56% of DCF and 2.41 ± 1.01% of SMX, respectively. Autoclaving leads to the destruction of the bacteria (Berns et al. 2008); therefore, the adsorption of the pharmaceuticals to damaged cell structures might not fully reflect the process that is characteristic of non-autoclaved cultures. The conclusion that adsorption of SMX and DCF onto biomass is negligible, should be confirmed in further investigations by using a different strain, similar to the tested one, but not able to degrade or transform pharmaceuticals. In many research studies, the adsorption of different pharmaceuticals was tested by using autoclaved biomass and our results agreed with those obtained by Yu et al. (2011), who found that SMX is weakly adsorbed to the microorganisms. Herzog et al. (2013) observed that adsorption of SMX onto autoclaved biomass of strains isolated from sulfamethoxazole-acclimated activated sludge was negligible. Moreira et al. (2018) didn't find any adsorption of DCF onto the heat-killed bacterial strain *Labrys portucalensis* F11.

The adsorption process may influence the biodegradation of pharmaceuticals. The affinity of SMX and DCF to solids was usually determined for activated sludge. Several approaches were used, based on e.g. the solid-water distribution coefficient (K_d) of the respective compound. Carballa et al. (2008) determined the log K_d values ranging from 1.3 to 2.2 for DCF and 0.8 to 1.8 for SMX. These values were significantly lower than those obtained for the musk fragrances or estrogens, indicating that these compounds do not adsorb onto sludge to an appreciable extent.

Considering the above information, it can be assumed that in our investigations the MG7 strain is involved in SMX and DCF removal. Some studies have reported on the microbial removal of SMX and DCF. Jiang et al. (2014) described the strain *Pseudomonas psychrophila* HA-4, which was able to remove 34.30% of 100 mg/l of SMX at 10°C after 192 h of incubation. Aissaoui et al. (2017) demonstrated that strains identified as *Pseudomonas* sp. and *Arthrobacter nicotianae* were able to remove 34.27% and 32.95% of 3 mg/l of SMX, respectively after 48 h of incubation. Biodegradation of DCF was mainly observed in white-rot fungi. Marco-Urrea et al. (2010) investigated the transformation of DCF by *Trametes versicolor*, and after 4 h, the efficiency of removal was 94%, when the initial concentration of DCF was 10 mg/l, but the adsorption on the surface of the fungus cells was significant. The efficiency of DCF removal was also investigated in activated sludge communities and microbial transformation did not lead to complete degradation (Kosjek et al. 2009). Langenhoff et al. (2013) demonstrated the ability of cultures enriched from activated sludge to biodegrade DCF (from 50 to 300 mg/l) over three weeks. However, the responsible strain(s) were not identified. Bessa et al. (2017) demonstrated that strain *Brevibacterium* sp. D4 was able to biodegrade 35% of 10 mg/l of DCF as the sole carbon source.

In our investigation, only SMX and DCF removal was measured. However, further research should focus on the determination of intermediates and/or stable metabolites and the description of the possible degradation pathway. Only a few studies reported information on the enzymes and different reaction mechanisms involved in SMX breakdown. Ricken et al. (2013) described the molecular mechanism in *Microbacterium* sp. strain BR1 for the breakdown of SMX. In this bacterium, degradation was initiated by ipso-hydroxylation and SMX fragmentation was observed. The NADH-dependent hydroxylation of the carbon atom attached to the sulfonyl group resulted in the release of sulfite, 3-amino-5-methylisoxazole, and benzoquinone-imine. The last compound was transformed to 4-aminophenol. Mulla et al. (2018) reported that *Chrobactrum* sp., *Labrys* sp. and *Gordonia* sp. were able to degrade 45.2%, 62.2% and 51.4% of SMX, respectively (the initial concentration was 5 mg/l). In all three bacterial cultures, SMX was sequentially converted into 3-amino-5-methylisoxazole, 4-aminophenol, and hydroquinone. Larcher and Yargeau (2011) reported that the arylamine N-acetyltransferase, which shows high specificity for aromatic amines, is involved in the degradation of SMX in *P. aeruginosa* and *Rhodococcus* species. Hu et al. (2017) proposed the pathways of SMX degradation by *Enterobacter cloacae* strain T2. The initial metabolites of SMX biodegradation follow four pathways: methylation, substitution by hydroxyl and double bond oxidation, desulphurization and acetylation. N-(4-Hydroxyphenyl)acetamide might be attributed to hydroxylation of N-phenylacetamide; N-phenylacetamide was also converted to benzoic and phenylacetic acid through

other pathways. The final process was the degradation of the benzene ring. Compounds containing a benzene ring are hydroxylated to form catechol and its structural isomers under catalysis of dioxygenases.

Despite the existing reports on the microbial degradation of DCF, there are still knowledge gaps related to the degradation process. Gröning et al. (2007) observed the removal of DCF in a concentration of 3–35 μM in a river sediment, with transient accumulation of a major metabolite, the pbenzoquinone imine derivative of 5-hydroxy-DCF, which is further abiotically adsorbed on the biofilm and this reveals a lack of biodegradation potential for this intermediate compound. Moreira et al. (2018) reported that the bacterial strain *L. portucalensis* F11 degraded 70% of 34 μM of DCF, supplied as the sole carbon source after 30 days of the culture; while its complete degradation was achieved *via* co-metabolism with acetate after 25 days. DCF degradation was mainly followed by hydroxylation reactions. Hydroxylation of DCF resulted in the formation of four isomers, from which it was possible to elucidate the structure of 4- and 5-hydroxy-DCF. Further degradation can occur through different pathways involved, e.g., methylation or decarboxylation. The formation of benzoquinone imine species seems to be a central step in the degradation pathway.

In our investigations, the additional carbon source used in the biodegradation tests of DCF and SMX was phenol. In plants, microbial endophytes can be exposed to secondary metabolites as phenolic compounds (Balasundram et al. 2006). Phenolic compounds are usually produced as a defense compounds against an attack on the plant tissue or in a stressful environment (e.g. unfavorable temperature, light, pH conditions or presence of xenobiotics in the soil). The basic structure of a phenolic compound is a benzene ring with hydroxyl substituent(s) (Cohen and Kennedy, 2010). Plant-microbial interaction requires sustained and prolonged reactions of the endophytes against the host defense mechanisms. Therefore, the selective pressure could be exerted on endobacterial communities and the growth of microorganisms that possess the specific enzymatic systems could be promoted.

In our investigations, in the presence of phenol as the additional carbon source, the efficient removal of pharmaceuticals, particularly SMX, was determined. Phenol was added to cultures gradually, to give a final concentration of 100 mg/l. An increase in optical density of the suspension was observed. However, the growth of biomass of the MG7 strain was more extensive in cultures containing SMX. The higher degradation of pharmaceuticals could be explained by adaptive mechanisms in microorganisms exposed to phenol. There is a correlation between an increase in membrane fatty acids saturation and the increased tolerance towards the toxic compounds in phenol-degrading strains (Mrozik et al. 2010). Among the adaptive mechanisms, changes in the fatty acid composition of membrane lipids are the most important reactions of bacteria to membrane-active substances (Neumann et al. 2004). Owing to this, the bacterial membranes become more resistant to the fluidizing action of aromatic compounds, which allows the cells to survive in the contaminated sites (Salar et al. 2014). The other adaptive mechanisms might involve changes in the following: membrane protein pattern, energy metabolism, cell morphology, the cell wall as well as the vesicle formation and transformation or degradation of xenobiotics (Heipieper et al. 2007; Schrewe et al. 2013). The higher degradation of SMX and DCF in the presence of phenol could also be explained by co-metabolic interaction. Non-growth substrates (pharmaceuticals), which have a similar structure to growth substrates (phenol) can bind to enzymes and be transformed due to their non-specific activity (Fatta-Kassinos et al. 2010). Most pathways on the degradation of aromatics go through oxygenation reactions catalyzed by mono- or dioxygenases, to convert these compounds to dihydroxy aromatic intermediates. As was mentioned before, the final step in SMX breakdown is the degradation of the benzene ring and compounds containing a benzene ring are hydroxylated to form catechol, which is also produced by the oxidation of phenol. The dihydroxyl compounds produced subsequently undergo a ring fission by dioxygenases, to produce intermediates of the Kreb's cycle (Cao et al. 2009)

The bacterial endophytes that can be grown in the laboratory consist only a small fraction of the total diversity that exists in the plant tissues. The literature generally suggests that only 0.001% to 1% of the endophytes present in plant tissues are cultivable (Torsvik and Øvreås, 2002; Alain and Querellou 2009). Therefore, the response of all endophytic bacterial communities to the pressure caused by the presence of DCF and SMX in wastewater should also be taken into consideration. In this study, the biodiversity of endophytic bacterial communities of reed canary grass, non-exposed and exposed to DCF and SMX was assessed using PCR-DGGE based on DNA extracted from root tissues. Analyzing the results, the values of Shannon biodiversity index were comparable for all samples. However, the highest value was calculated for sample obtained from columns B1-3, in which plants were exposed to DCF and SMX. This result is surprising because it was expected that pharmaceuticals, in particular SMX, would have exerted a selective pressure on endophytic bacterial communities and a loss of biodiversity should be observed. Taking into consideration the fingerprint obtained from DGGE separation of 16S rRNA gene fragments, different band patterns among the samples were observed. This situation may be associated with the adaptation of the

endobacterial communities to the presence of pharmaceuticals. It should also be mentioned that a similar difference in bands' composition was not clearly visible in the samples obtained from columns D1-3, in which plants also were exposed to DCF and SMX but in a different time regime. The fingerprints obtained for samples from columns in which plants were exposed to pharmaceuticals showed some similarities. However, it seems that the diversity of endophytic bacteria communities depends on both the presence of DCF and SMX and frequency of supply of the constructed wetland systems with the wastewater. Therefore, further experiments should be conducted to confirm this hypothesis.

Conclusions

Constructed wetland systems are a promising technology for treating wastewater containing micropollutants, including residues of pharmaceuticals. The bacterial endophytes can be exposed to secondary metabolites in plant tissues. Therefore, they can possess the potential to transform or degrade aromatic structures, including pharmaceuticals. When the initial concentration was 2 mg/l, the strain MG7 identified as *Microbacterium flavescens* was able to remove approximately 15% of DCF and 9% of SMX after 20 days of incubation in monosubstrate culture. A decrease in the optical density of the cultures was observed, which indicated that the applied carbon source didn't provide enough energy for bacterial growth and proliferation. The adsorption of DCF and SMX onto autoclaved bacteria was negligible. However, this observation should be confirmed with other methods. In the presence of phenol as the additional carbon source, the strain MG7 removed approximately 35% of DCF and 61% of SMX, when the initial concentration was also 2 mg/l. The adaptive mechanisms in microorganisms exposed to phenol (i.e. changes in the composition of membrane lipids) or a co-metabolic mechanism, when non-growth substrates can be transformed by non-specific enzymes could participate in this phenomenon. Both the presence of pharmaceuticals and frequency of supply of CWs with the contaminated wastewater affected the diversity of the whole endophytic bacterial communities. However, further experiments should be conducted to confirm this hypothesis. The results obtained suggest the capability of the strain MG7 for degradation of DCF and SMX. However, further research should focus on the determination of intermediates and/or stable metabolites and assessment of the strains' potential for bioaugmentation in CWs.

Acknowledgement

The project was supported by Grant BKM 08/080/BKM16/0039 and Grant BK 08/080/BK_18/0054 from the Ministry of Science and Higher Education.

Literature

Acuña V, Ginebreda A, Mor JR, Petrovic M, Sabater S, Sumpter J, Barceló D. 2015. Balancing the health benefits and environmental risks of pharmaceuticals: Diclofenac as an example. Environ Int. 85:327–333.

Afzal M, Khan QM, Sessitsch A. 2014. Endophytic bacteria: prospects and applications for the phytoremediation of organic pollutants. Chemosphere. 117:232–242.

Aissaoui S, Sifour M, Ouled-Haddar H, Sghaier H, Jamoussi B. 2017. Microbial removal of sulfamethoxazole by pure bacterial cultures isolated from the environment. JEMS. 8(6):2147–2153.

Alain K, Querellou J. 2009. Cultivating the uncultured: limits, advances and future challenges. Extremophiles. 13:583–594.

Balasundram N, Sundram K, Samman S. 2006. Phenolic compounds in plants and agri-industrial by-products: Antioxidant activity, occurrence, and potential uses. Food Chem. 99(1):191–203.

Baran W, Adamek E, Ziemiańska J, Sobczak A. 2011. Effects of the presence of sulfonamides in the environment and their influence on human health. J Hazard Mater. 196:1–15.

Barbosa MO, Moreira NF, Ribeiro AR, Pereira MF, Silva AM. 2016. Occurrence and removal of organic micropollutants: An overview of the watch list of EU Decision 2015/495. Water Res. 94:57–79.

Berns AE, Philipp H, Narres H-D, Burauel P, Vereecken H, Tappe W. 2008. Effect of gamma-sterilization and autoclaving on soil organic matter structure as studied by solid state NMR, UV and fluorescence spectroscopy. Eur J Soil Sci. 59:540–550.

Bessa VS, Moreira IS, Tiritan ME, Castro PML. 2017. Enrichment of bacterial strains for the biodegradation of diclofenac and carbamazepine from activated sludge. Int Biodeter Biodegr. 120:135–142.

Carballa M, Fink G, Omil F, Lema JM, Ternes T. 2008. Determination of the solid – water distribution coefficient (K_d) for pharmaceuticals, estrogens and musk fragrances in digested sludge. Water Res. 42:287–295.

Cao B, Nagarajan K, Loh K-C. 2009. Biodegradation of aromatic compounds: current status and opportunities for biomolecular approaches. Appl Microbiol Biotechnol. 85:207–228.

Cohen SD, Kennedy JA. 2010. Plant metabolism and the environment: Implications for managing phenolics. Crit Rev Food Sci Nutr. 50:620–643.

Danevčič T, Vezjak MB, Tabor M, Zorec M, Stopar D. 2016. Prodigiosin induces autolysins in actively grown Bacillus subtilis cells. Front Microbiol. 7:27.

Eevers N, Hawthorne JR, White JC, Vangronsveld J, Weyens N. 2016. Exposure of *Cucurbita pepo* to DDE-contamination alters the endophytic community: A cultivation dependent vs. a cultivation independent approach. Environ Pollut. 209:147–154.

Fatta-Kassinos D, Bester K, Kümmerer K. 2010. Xenobiotics in the urban water cycle. Environmental Pollution 16. Heidelberg (Germany): Springer Science and Business Media B.V.

Felis E, Sochacki A, Magiera S. 2016. Degradation of benzotriazole and benzothiazole in treatment wetlands and by artificial sunlight. Water Res. 104:441–448.

Gao P, Mao D, Luo Y, Wang L, Xu B, Xu L. 2012. Occurrence of sulfonamide and tetracycline-resistant bacteria and resistance genes in aquaculture environment. Water Res. 46:2355–2364.

Gröning J, Held C, Garten C, Claußnitzer U, Kaschabek SR, Schlömann M. 2007. Transformation of diclofenac by the indigenous microflora of river sediments and identification of a major intermediate. Chemosphere. 69:509–516.

Heipieper HJ, Neumann G, Cornelissen S, Meinhardt F. 2007. Solvent-tolerant bacteria for biotransformations in two-phase fermentation systems. Appl Microbiol Biotechnol. 74:961–973.

Herzog B, Lemmer H, Lemmer H, Müller E. 2013. Characterization of pure cultures isolated from sulfamethoxazole-acclimated

activated sludge with respect to taxonomic identification and sulfamethoxazole biodegradation potential. BMC Microbiol. 13:276.
Hijosa-Valsero M, Reyes-Contreras C, Domínguez C, Bécares E, Bayona JM. 2016. Behaviour of pharmaceuticals and personal care products in constructed wetland compartments: Influent, effluent, pore water, substrate and plant roots. Chemosphere. 145:508–517.
Hirsch R, Ternes T, Haberer K, Kratz K-L. 1999. Occurrence of antibiotics in the aquatic environment. Sci. Total Environ. 225:109–118.
Hu S, Hu H, Li W, Ke Y,Li M, Zhao Y. 2017. Enhanced sulfamethoxazole degradation in soil by immobilized sulfamethoxazole-degrading microbes on bagasse. RSC Adv. 7:55240–55248.
Jiang B, Li A, Cui D, Cai R, Ma F, Wang Y. 2014. Biodegradation and metabolic pathway of sulfamethoxazole by *Pseudomonas psychrophila* HA-4, a newly isolated cold-adapted sulfamethoxazole-degrading bacterium. Appl Microbiol Biotechnol. 98(10):4671–4681.
Kim D-W, Heinze TM, Kim B-S, Schnackenberg LK, Woodling KA, John B, Sutherland JB. 2011. Modification of norfloxacin by a *Microbacterium* sp. strain isolated from a wastewater treatment plant. Appl Environ Microbiol. 77(17):6100–6108.
Kosjek T, Heath E, Pérez S, Petrović M, Barceló D. 2009. Metabolism studies of diclofenac and clofibric acid in activated sludge bioreactors using liquid chromatography with quadrupole – time-of-flight mass spectrometry. J Hydrol. 372:109–117.
Langenhoff A, Inderfurth N, Veuskens T, Schraa G, Blokland M, Kujawa-Roeleveld K, Rijnaarts H. 2013. Microbial removal of the pharmaceutical compounds ibuprofen and diclofenac from wastewater. BioMed Research Int. 2013:325806.
Larcher S, Yargeau V. 2011. Biodegradation of sulfamethoxazole by individual and mixed bacteria. Appl Microbiol Biotechnol. 91(1):211–218.
Maier RM, Pepper IL, Gerba CP. 2000. Environmental Microbiology. San Diego (USA): Academic Press.
Marco-Urrea E, Pérez-Trujillo M, Cruz-Morato C, Caminal G, Vicent T. 2010. Degradation of the drug sodium diclofenac by *Trametes versicolor* pellets and identification of some intermediates by NMR. J Hazard Mater. 176:836–842.
Moreira IS, Bessa VS, Murgolo S, Piccirillo C, Mascolo G, Castroa PML. 2018. Biodegradation of diclofenac by the bacterial strain *Labrys portucalensis* F11. Ecotox Environ Safe. 152:104–113.
Mrozik A, Cycon M, Piotrowska-Seget Z. 2010. Changes of FAME profiles as a marker of phenol degradation in different soils inoculated with *Pseudomonas* sp. CF600. Int Biodeter Biodegr. 64(1):86–96.
Mulla SI, Hu A, Sun Q, Li J, Suanon F, Ashfaq M, Yu CP. 2018. Biodegradation of sulfamethoxazole in bacteria from three different origins. J Environ Manage. 206:93–102.
Muyzer G, De Waal EC, Uitierlnden AG. 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl Environ Microbiol. 59:695–700.
Neumann G, Teras R, Monson L, Kivisaar M, Schauer F, Heipieper HJ. 2004. Simultaneous degradation of atrazine and phenol by *Pseudomonas* sp. strain ADP: effects of toxicity and adaptation. Appl Environ Microbiol. 70(4):1907–1912.
Nopens I, Capalozza C, Vanrolleghem PA. 2001. Stability analysis of a synthetic municipal wastewater. Department of Applied Mathematics Biometrics and Process Control. Gent (Belgium): University of Gent.
Nowrotek M, Sochacki A, Felis E, Miksch K. 2016. Removal of diclofenac and sulfamethoxazole from synthetic municipal waste water in microcosm downflow constructed wetlands: start-up results. Int J Phytoremediat. 18(2):157–163.
Ricken B, Corvini PFX, Cichocka D, Parisi M, Lenz M, Wyss D, Martínez-Lavanchy PM, Müller JA, Shahgaldian P, Tulli LG, et al. 2013. Ipso-hydroxylation and subsequent fragmentation – a novel microbial strategy to eliminate sulfonamide antibiotics. Appl Environ Microbiol. 79(18):5550–5558.
Ricken B, Kolvenbach BA, Bergesch C, Benndorf D, Kroll K, Strnad H, Vlček Č, Adaixo R, Hammes F, Shahgaldian P, et al. 2017. $FMNH_2$-dependent monooxygenases initiate catabolism of sulfonamides in *Microbacterium* sp. strain BR1 subsisting on sulfonamide antibiotics. Sci Rep. 7(1):15783.
Salar RK, Gahlawat SK, Siwach P, Duhan JS. 2014. Biotechnology: prospects and applications. Heidelberg (Germany): Springer Science and Business Media B.V.
Sauvêtre A, Schröder P. 2015. Uptake of carbamazepine by rhizomes and endophytic bacteria of *Phragmites australis*. Front Plant Sci. 6:83.
Schaider LA, Rudel RA, Ackerman JM, Dunagan SC, Brody JG. 2014. Pharmaceuticals, perfluorosurfactants, and other organic wastewater compounds in public drinking water wells in a shallow sand and gravel aquifer. Sci Total Environ. 468–469:384–393.
Schrewe M, Julsing MK, Bühler B, Schmid A. 2013. Whole-cell biocatalysis for selective and productive C-O functional group introduction and modification. Chem Soc Rev. 42:6346–6377.
Schwaiger J, Ferling H, Mallow U, Wintermayr H, Negele DR. 2004. Toxic effects of the non-steroidal anti-inflammatory drug diclofenac Part I: Histopathological alterations and bioaccumulation in rainbow trout. Aquat Toxicol. 68:141–150.
Shannon CE, Weaver W. 1963. The Mathematical Theory of Communication. Urbana (USA): University of Illinois Press.
Shelef O, Gross A, Rachmilevitch S. 2013. Role of plants in a constructed wetland: current and new perspectives. Water 5: 05–419.
Siciliano SD, Fortin N, Mihoc A, Wisse G, Labelle S, Beaumier D, Ouellette D, Roy R, Whyte LG, Banks K, et al. 2001. Selection of specific endophytic bacterial genotypes by plants in response to soil contamination. Appl Environ Microbiol. 67:2469–2475.
Sui Q, Cao X, Lu S, Zhao W, Qiu Z, Yu G. 2015. Occurrence, sources and fate of pharmaceuticals and personal care products in the groundwater: A review. Emerging Contaminants. 1:14–24.
Sukul P, Spiteller M. 2006. Sulfonamides in the environment as veterinary drugs. Rev Environ Contam Toxicol. 187:67–101.
Syranidou E, Christofilopoulos S, Gkavrou G, Thijs S, Weyens N, Vangronsveld J, Kalogerakis N. 2016. Exploitation of endophytic bacteria to enhance the phytoremediation potential of the wetland helophyte *Juncus acutus*. Front Microbiol. 7:1016.
Torsvik V, Øvreås L. 2002. Microbial diversity and function in soil: from genes to ecosystems. Curr Opin in Microbiol. 5:240–245.
Yin J, Sun L, Dong Y, Chi X, Zhu W, Qi S-H, Gao H. 2013. Expression of *bla*A underlies unexpected ampicillin-induced cell lysis of *Shewanella oneidensis*. PLoS One 8(3):e60460.
Yu TH, Lin AY, Panchangam SC, Hong PK, Yang PY, Lin CF. 2011. Biodegradation and bio-sorption of antibiotics and non-steroidal anti-inflammatory drugs using immobilized cell process. Chemosphere. 84(9):1216–1222.
Verlicchi P, Zambello E. 2014. How efficient are constructed wetlands in removing pharmaceuticals from untreated and treated urban wastewaters? A review. Sci Total Environ. 470–471:1281–1306.
Vieno N, Sillanpää M. 2014. Fate of diclofenac in municipal wastewater treatment plant – A review. Environ. Int. 69:28–39.
Weyens N, van Der Lelie D, Taghavi S, Vangronsveld J. 2009. Phytoremediation: plant-endophyte partnerships take the challenge. Curr Opin Biotechnol. 20:1–7.
Zhang DQ, Jinadasa KBSN, Gersberg RM, Liu Y, Ng WJ, Tan SK. 2014. Application of constructed wetlands for wastewater treatment in developing countries – A review of recent developments (2000–2013). J Environ Manage.141:116–141.

Polish Journal of Microbiology
2018, Vol. 67, No 3, 333–338
https://doi.org/10.21307/pjm-2018-040

ORIGINAL PAPER

Carbapenem-resistant *Acinetobacter baumannii* from Air and Patients of Intensive Care Units

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Submitted 28 October 2017, revised 29 December 2017, accepted 7 July 2018

Abstract

To understand the molecular epidemiology and antibiotic resistance of air and clinical isolates of *Acinetobacter baumannii*, the intensive care unit settings of a hospital in Northern China were surveyed in 2014. Twenty non-duplicate *A. baumannii* isolates were obtained from patients and five isolates of airborne *A. baumannii* were obtained from the wards' corridors. Pulsed field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) were used to analyze the homology relationships of isolates. Resistance and resistance genes were detected by drug susceptibility test and PCR. The results demonstrated that all isolates can be classified into eight PFGE types and four sequence types (ST208, ST195, ST369 and ST530). A pair of isolates from patients (TAaba004) and from the air (TAaba012) that share 100% similarity in PFGE was identified, indicating that air might be a potential and important transmission route for *A. baumannii*. More than 80% of the isolates were resistant to carbapenems and aminoglycoside antibiotics. Twenty-four isolates, which were resistant to carbapenems, carried the $bla_{\text{OXA-23-like}}$ gene. The data indicated that air might be an alternative way for the transmission of *A. baumannii*. Hospitals should pay more attention to this route, and design new measures accordingly.

Key words: *Acinetobacter baumannii*, molecular epidemiology, antibiotic resistance, airborne, nosocomial infection

Introduction

Outbreaks caused by multidrug resistant *Acinetobacter baumannii* strains have been intensively documented worldwide (Zarrilli et al. 2013). With the increasing number of carbapenem-resistant *A. baumannii*, outbreaks of *A. baumannii* were expanding. One data showed that resistance to carbapenem increased from 1.0% in 2003 to 58.0% in 2008 in Detroit Medical Center health system (Reddy et al. 2010). In China, *A. baumannii* has recently become an important pathogen causing the nosocomial infection in hospitals. The infection rate of *A. baumannii* is always in the top three in total clinical isolates of Gram-negative bacilli, second only to *Escherichia coli* and *Klebsiella pneumoniae*. In 2014, *A. baumannii* isolated from non-fermenting Gram-negative bacilli was always in the first place in clinical tests (Wang et al. 2013; Hu et al. 2014; Hu et al. 2015).

The analysis results of multilocus sequence typing (MLST) demonstrated that multiple STs were prevalent in the outbreak regions. Ying reported that ST208 was the most prevalent, followed by ST191 and ST729 in the 398 isolates collected in seven regions of South China in 2012 (Ying et al. 2015). Through the whole genome sequencing, it was found that $bla_{\text{OXA-23}}$-producing *A. baumannii* ST208 strains emerged and rapidly spread in Hangzhou First People's Hospital (Chen et al. 2018). Just recently, Jeon et al. (2018) reported that sequence type (ST) 191 was the predominant clone, followed by the ST208 and ST369 in the hospital in Korea.

At present, *A. baumannii* strains were mainly isolated from the clinical specimens, as blood, urine, tissues, and sputum (Bogaerts et al. 2006; Zong et al. 2008; Mendes et al. 2009; Yang et al. 2010; Chen et al. 2013). In our laboratory, we have been focused on the epidemiology of *A. baumannii* isolates from sputum (Jiang et al. 2013; Jiang et al. 2014). However, some

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reports have shown that *A. baumannii* can survive and transmit in the air (Allen et al. 1987; Obbard et al. 2003). To date, the homology relationship between the airborne *A. baumannii* isolates from hospital air and those isolated from patients has not been extensively studied. In this study, twenty-five isolates of non-duplicate *A. baumannii* were obtained from patients and the air of wards' corridors during May – Jun. and Oct. – Nov. of 2014. The molecular epidemiological characterization of the isolated *A. baumannii*, such as drug resistance, and types of resistance genes, was compared to understand the relationship between the airborne *A. baumannii* and those isolated from patients.

Experimental

Materials and Methods

Ethical approval. Samples of patients were acquired during a routine checkup by medical staffs. The study was approved by the Ethics Committee of Tai'an City Central Hospital and carried out in accordance with the approved guidelines.

Source of the isolates. From May 12 to Jun. 5 and Oct. 11 to Nov. 15, 2014, *A. baumannii* isolates were obtained from patients hospitalized in four different wards, including the intensive care unit (ICU), emergency intensive care unit (EICU), respiratory intensive care unit (RICU) and geriatrics ward (GW). Meanwhile, airborne *A. baumannii* isolates were obtained from the air of the wards and adjacent corridors using the Andersen 6-stage air sampler. Each stage of the sampler has a plate with 400 holes of uniform diameter, according to the method described in previous studies (Andersen et al. 1958; Obbard et al. 2003). In brief, the sampler was placed in the center of wards and corridors (closed) at a height of 1.5 m above the ground. Airborne *A. baumannii* was collected at a calibrated flow rate of 28.3 l/min for 20 min, and MacConkey agar (Oxoid, Basingstoke, England) was used as the sampling medium. At each sampling site, four duplicate air samples were collected once per week. The number of colonies was about 30–300 in each stage. Temperature and relative humidity were recorded throughout the sampling periods (temperature 20–25°C; relative humidity 73–83%).

Bacterial identification and drug susceptibility test. Bacterial identification was performed by using an VITEK-2 bacterial instrument (BioMerieux, Lyons, France) following the operation instruction. Drug susceptibility test was performed by two different methods: the sensitivity of tigecycline and polymyxin B was detected by the Etest method (AB Biodisk, Solna, Sweden); the sensitivity of the remaining fourteen antibiotics was detected using the minimal inhibitory concentration method. The susceptibility testing result of tigecycline was explained according to Food and Drug Administration (FDA) guideline (http://www.fda.org.uk/sitemap.aspx). The criteria of the susceptibility of other agents were adapted from the Clinical and Laboratory Standards Institute (CLSI) (http://clsi.org/standards/). The criteria of multidrug resistant (MDR) *A. baumannii*, extensively drug-resistant (XDR) *A. baumannii* and pandrug-resistant (PDR) *A. baumannii* were referred to the previously described (Magiorakos et al. 2012).

Pulsed field gel electrophoresis (PFGE). Clonal relatedness of 25 *A. baumannii* strains isolated in this study was determined by PFGE (Ribot et al. 2006). Briefly, the bacterial chromosomal DNA was digested with 60 U of *ApaI* (Takara, Dalian, China) in a 37°C. *Salmonella* serotype Braenderup strain (H9812) was digested with *XbaI* (Takara, Dalian, China) and used as the molecular weight standard, the DNA fragments were separated on agarose gel using a clamped homogeneous electric field electrophoresis-Mapper XA system (Bio-Rad, California, USA). The PFGE images were analyzed using the Gel Doc software (Bio-Rad, California, USA) and the genetic relatedness of the *A. baumannii* isolates were evaluated using the BioNumerics software (Applied Maths, Sint-Martens-Latem, Belgium). The cutoff level of 85% was set to define clonal clustering of the PFGE types.

Multilocus sequence typing (MLST). The sequence types (STs) of *A. baumannii* strains were determined by MLST using the previously described method (Bartual

Table I
Susceptibility profiles of 20 clinical *A. baumannii* isolates.

Antibiotics	Susceptible		Resistant	
	Number	Rate (%)	Number	Rate (%)
Amikacin	3	15.0%	17	85.0%
Ceftazidime	0	0.0%	20	100%
Ciprofloxacin	0	0.0%	20	100%
Levofloxacin	0	0.0%	20	100%
Gentamicin	0	0.0%	20	100%
Tobramycin	3	15.0%	17	85.0%
Ceftriaxone	0	0.0%	20	100%
Cefotaxime	0	0.0%	20	100%
Cefepime	0	0.0%	20	100%
Imipenem	0	0.0%	20	100%
Meropenem	0	0.0%	20	100%
Trimethoprim/ sulfamethoxazole	6	30.0%	14	100%
Piperacillin/ tazobactam	0	0.0%	20	100%
Tigecycline	20	100%	0	0.0%
Polymyxin B	20	100%	0	0.0%

Table II
Susceptibility profiles of the airborne and clinical strains with the highest similarity.

Strain / Antibiotics	TAaba004	TAaba012
Amikacin	R	R
Ceftazidime	R	R
Ciprofloxacin	R	R
Levofloxacin	R	R
Gentamicin	R	R
Tobramycin	R	R
Ceftriaxone	R	R
cefotaxime	R	R
Cefepime	R	R
Imipenem	R	R
Meropenem	R	R
Trimethoprim/sulfamethoxazole	R	R
Piperacillin/tazobactam	R	R
Tigecycline	S	S
Polymyxin B	S	S

Note: "R" indicates resistant, "S" indicates susceptible.

et al. 2005). In brief, the internal fragments of seven housekeeping genes, including citrate synthase (gltA), DNA gyrase subunit B (gyrB), glucose dehydrogenase B (gdhB), homologous recombination factor (recA), 60-kDa chaperonin (cpn60), glucose-6-phosphate isomerase (gpi) and RNA polymerase σ70 factor (rpoD) were amplified by PCR. The positive sequences were further analyzed using the *A. baumannii* PubMLST database (http://pubmlst.org/abaumannii).

Drug resistance genes. Bacterial DNA were extracted and the carbapenemase genes, including $bla_{\text{OXA-23-like}}$, $bla_{\text{OXA-24-like}}$, $bla_{\text{OXA-48}}$, $bla_{\text{OXA-50}}$, $bla_{\text{OXA-51-like}}$, $bla_{\text{OXA-55}}$, $bla_{\text{OXA-58-like}}$, $bla_{\text{OXA-60}}$, and $bla_{\text{OXA-64}}$, the 16S rRNA methylase genes *armA* and *rmtB* were amplified by PCR as previously described (Shen et al. 2008; Yang et al. 2011). The positive bands were sequenced and then compared to the Genbank database (Jiang et al. 2014).

Results

Isolation of *A. baumannii* strains. Twenty *A. baumannii* clinical strains were isolated from 356 patients and five strains were isolated from 4 out of 64 air samples in duplicate during this experimental period (8 weeks). Wherein two *A. baumannii* strains (TAaba015 and TAaba014) were isolated from the same air sample from ICU corridor in 08.11.2014.

Drug susceptibility patterns. A total of twenty-five non-duplicate *A. baumannii* isolates were obtained, including twenty from the clinical samples and five from the adjacent corridors of the wards. Fifteen commonly used antibiotics (amikacin, ceftazidime, ciprofloxacin, levofloxacin, gentamicin, tobramycin, ceftriaxone, cefotaxime, cefepime, imipenem, meropenem, trimethoprim/sulfamethoxazole, piperacillin/tazobactam, tigecycline, and polymyxin B) were selected for drug susceptibility test. The result showed that only one out of the five airborne isolates (TAaba013) was non-MDR *A. baumannii*, which was only resistant to ceftazidime, ceftriaxone, cefotaxime, and cefepime. All twenty clinical isolates were MDR *A. baumannii*, and susceptible to tigecycline and polymyxin B.

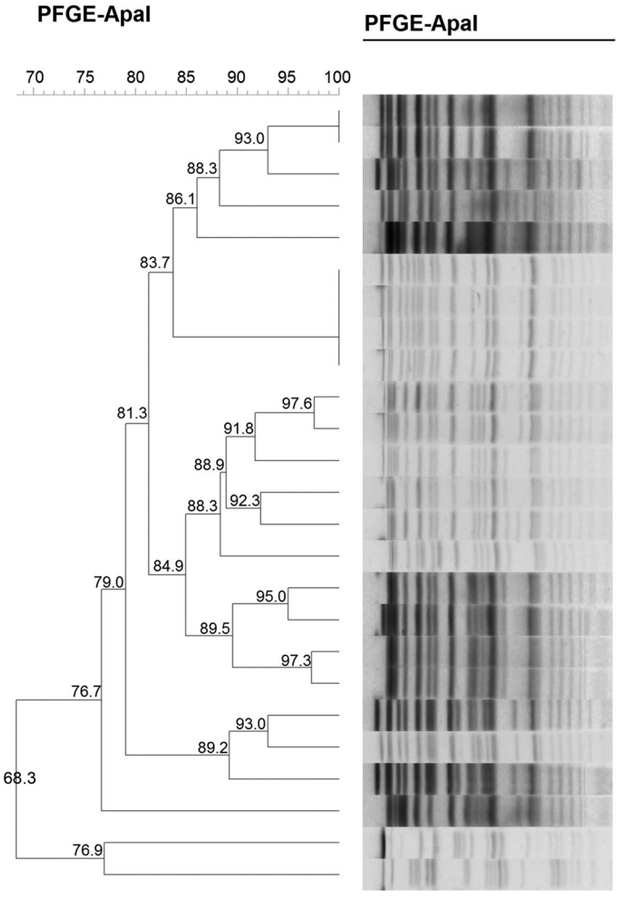
Drug resistance genes. All isolates had the $bla_{\text{OXA-51-like}}$ resistant gene, and twenty-four had the $bla_{\text{OXA-23-like}}$ gene except one airborne isolate (TAaba014). However, other seven resistance genes of carbapenemases were not detected in this study. These results indicated that $bla_{\text{OXA-23-like}}$ gene was responsible for the carbapenemase resistance. In addition, twenty isolates carried the 16S rRNA methylase gene *armA*. No *rmtB* gene was detected in all isolates.

PFGE and MLST. As shown in Fig. 1, the isolates can be clustered into eight PFGE types (A-H) and four STs (ST208, ST369, ST195 and ST530). One airborne isolate (TAaba012) had the same PFGE and ST types as one clinical isolates (TAaba004). They were isolated from the same ward (EICU) during the same period (May – Jun 2014). Similarly, in another ward (intensive care unit, ICU), one airborne isolate (TAaba008) was found to be closely related to three clinical isolates (TAaba002, TAaba009, and TAaba011) in terms of PFGE and ST types (type D and ST208, respectively).

In the second sampling period (Oct. 11 to Nov. 15, 2014), we collected three non-reduplicate airborne isolates (TAaba013, TAaba014 and TAaba015). We did not find any clinical isolates with the same PFGE and ST types. In particular, the PFGE and ST types of TAaba013 were different from any isolates obtained in this study.

Discussion

A. baumannii is a predominant cause of nosocomial infections, and the organism has been detected in water, soil, and on the surface of human body. A recent study has shown that some *A. baumannii* environmental isolates are able to survive in tap, normal saline, and distilled water with a wide range of pH and temperature for a long period of time (Obeidat et al. 2014). It makes it harder for the prevention of *A. baumannii* spread in a nosocomial environment. *A. baumannii* is often transmitted through physical objects, like stethoscopes, computers, milk pumps, and headboards of beds. In this study, we investigated the genetic relationship of the *A. baumannii* isolates from the air and patients, and the possible transmission pathway through the air.



Isolates	PFGE types	ST	Ward distribution	Specimen sourses	Date of isolation	$bla_{OXA\text{-}like}$	$bla_{OXA\text{-}51\text{-}like}$	armA
TAaba004	A_1	369	Emergency Intensive Care Unit	Sputum	20.05.2014	+	+	+
TAaba012	A_1	369	Emergency Intensive Care Unit Corridor	Air	23.05.2014	+	+	+
TAaba003	A_2	369	Respiratory Intensive Care Unit	Sputum	17.05.2014	+	+	+
TAaba006	A_3	208	Intensive Care Unit	Sputum	23.05.2014	+	+	+
TAaba005	A_4	208	Respiratory Intensive Care Unit	Sputum	20.05.2014	+	+	–
TAaba016	B	195	Intensive Care Unit	Sputum	11.10.2014	+	+	+
TAaba017	B	195	Respiratory Intensive Care Unit	Sputum	27.10.2014	+	+	+
TAaba024	B	195	Intensive Care Unit	Sputum	10.11.2014	+	+	+
TAaba025	B	195	Intensive Care Unit	Wound	15.11.2014	+	+	+
TAaba020	C_1	208	Intensive Care Unit	Sputum	05.11.2014	+	+	+
TAaba021	C_2	208	Intensive Care Unit	Sputum	08.11.2014	+	+	+
TAaba023	C_3	208	Intensive Care Unit	Sputum	08.11.2014	+	+	+
TAaba018	C_4	208	Geriarics Department	Sputum	29.10.2014	+	+	+
TAaba022	C_5	208	Intensive Care Unit	Sputum	08.11.2014	+	+	–
TAaba015	C_6	369	Intensive Care Unit Corridor	Air	08.11.2014	+	+	+
TAaba002	D_1	208	Intensive Care Unit	Sputum	14.05.2014	+	+	+
TAaba011	D_2	208	Intensive Care Unit	Sputum	05.06.2014	+	+	+
TAaba008	D_3	208	Intensive Care Unit Corridor	Air	16.05.2014	+	+	+
TAaba009	D_4	208	Intensive Care Unit	Sputum	02.06.2014	+	+	+
TAaba001	E_1	369	Emergency Intensive Care Unit	Sputum	12.05.2014	+	+	+
TAaba019	E_2	369	Geriarics Department	Urine	31.10.2014	+	+	+
TAaba010	E_3	369	Emergency Intensive Care Unit	Sputum	01.06.2014	+	+	+
TAaba007	F	208	Intensive Care Unit	Sputum	31.05.2014	+	+	–
TAaba013	G	530	Respiratory Intensive Care Unit Corridor	Air	30.10.2014	+	+	–
TAaba014	H	208	Intensive Care Unit Corridor	Air	08.11.2014	–	+	–

Fig. 1. Dendrogram showing the PFGE and MLST profiles of the *A. baumannii* isolates. The dendogram was generated by the BioNumerics software. The sampling date, isolate number, ward and source, PFGE and ST types and resistance genes are shown for each isolate.

Our results show that one airborne *A. baumannii* (TAaba012) isolated in the EICU and one clinical isolate (TAaba004) isolated in the same ward shares 100% similarity in PFGE and MLST, indicating *A. baumannii* might be transmitted through air. Another airborne isolate (TAaba008) obtained in ICU was also found to be the same strain as three clinical isolates (TAaba002, TAaba009, and TAaba011). Based on the genetic relationship of the airborne and clinical isolates, we speculate that there could exist an air-mediated spread of this *A. baumannii* strain. Moreover, many studies demonstrated that airborne *A. baumannii* could be detected in ICU and airborne transmission of *A. baumannii* was possible (Munoz-Price et al. 2013; Spellberg et al. 2013; Yakupogullari et al. 2016).

A total of twenty clinical isolates were obtained during the sampling period of this study, but only one isolate was found in the air, indicating airborne transmission might be affected by many factors, such as length of hospital stay, humidity, temperature, etc. Since our dataset are relatively small, we cannot draw any exclusive conclusion. Moreover, because horizontal gene transfer that can jeopardize clonal strains, PFGE is no longer the most effective technique for *A. baumannii* typing (Salipante et al. 2015), and our data can only confirm that the same strain was isolated from air and the specimens from patients, which could suggest an alternative route of transmission through air. More systematic studies with a longer period of sampling should be constructed to better understand the airborne pathway.

Treatment of *A. baumannii* infections is particularly difficult because of the resistance to a broad range of antibiotics, especially with the increasing of resistant to carbapenems (Lee et al. 2012). Although mechanisms of resistance to carbapenems were very complex, resistance genes play an important role in this process. It has been reported that $bla_{\text{OXA-23-like}}$-producing *A. baumannii* were found worldwide (Le et al. 2008; Runnegar et al. 2010; Mosqueda et al. 2013). In this study, the analysis showed that all strains had $bla_{\text{OXA-51-like}}$ gene, one intrinsic gene in *A. baumannii*. Moreover, 96% strains (24/25) carried the $bla_{\text{OXA-23-like}}$ gene, except TAaba014. These results indicated that both genes were mainly responsible for the carbapenems resistance in this area, especially the $bla_{\text{OXA-23like}}$ gene and it was generally believed that $bla_{\text{OXA-23-like}}$ gene was responsible for carbapenem resistance in China (Ruan et al. 2010; Liu et al. 2015). Additionally, it has been reported that 16S rRNA methylase gene *armA* was closely related to the aminoglycosides resistance (Yokoyama et al. 2003), and 80% (20/25) strains had the 16S rRNA methylase gene *armA*. All of the strains showed the resistance to amikacin, tobramycin and gentamicin. Antibiotic resistance is a serious global public problem.

Taken together, in this study, we isolated twenty clinical *A. baumannii* from patients and five airborne *A. baumannii* from the ward environment. Out of the five airborne *A. baumannii,* one was found to have the same PFGE and ST type with one strain isolated from patients, indicating that the air may be an important and potential transmission way for *A. baumannii*. Considering the seriousness of nosocomial infections of *A. baumannii*, prophylactic strategies in controlling the air transmission of *A. baumannii* should be considered when new measures are designed for controlling nosocomial infections.

Acknowledgements

This research was supported by Natural Science Foundation of Shandong Province (ZR2013HM009).

Author contributions

M. Jiang and Z. Zhang performed the main experiment and wrote the manuscript. Y. Mu and N. Li analyzed data, Z. Zhang and S. Han reviewed the manuscript and approved it.

Conflict of interest statement

The authors declared there was no any conflict of interests.

Literature

Zarrilli R, Pournaras S, Giannouli M, Tsakris A. 2013. Global evolution of multidrug-resistant *Acinetobacter baumannii* clonal lineages. Int J Antimicrob Agents. 41:11–19.

Reddy T, Chopra T, Marchaim D, Pogue JM, Alangaden G, Salimnia H, Boikov D, Venezia SN, Akins R, Selman P, et al. 2010. Trends in antimicrobial resistance of *Acinetobacter baumannii* isolates from a metropolitan Detroit health system. Antimicrob Agents Ch. 54(5):2235–2238.

Hu F, Zhu D, Wang F, Jiang X, Xu Y, Zhang X. 2015. CHINET 2014 surveillance of bacterial resistance in China. Chin J Infect Chemother. 15:401–410.

Wang F, Zhu D, Hu F, Du Y. 2013. CHINET 2012 surveillance of bacterial resistance in China. Chin J Infect Chemother. 13:321–330.

Ying J, Lu JW, Zong L, Li AL, Pan RW, Cheng C, Li KP, Chen LQ, Ying JC, Tou HF, et al. 2016. Molecular epidemiology and characterization of genotypes of *Acinetobacter baumannii* isolates from regions of south China. Jpn J Infect Dis. 69(3):180–185.

Chen Q, Zhou JW, Fan JZ, Wu SH, Xu LH, Jiang Y, Ruan Z, Yu YS, Yu DJ, Wang XJ. 2018. Simultaneous emergence and rapid spread of three OXA-23 producing *Acinetobacter baumannii* ST208 strains in intensive care units confirmed by whole genome sequencing. Infect Genet Evol. 58:243–250.

Jeon H, Kim S, Kim MH, Kim SY, Nam D, Park SC, Park SH, Bae H, Lee HJ, Cho JH, et al. 2018. Molecular epidemiology of carbapenem-resistant *Acinetobacter baumannii* isolates from a Korean hospital that carry $bla_{\text{OXA-23}}$. Infect Genet Evol. 58:232–236.

Bogaerts P, Naas T, Wybo I, Bauraing C, Soetens O, Pierard D, Nordmann P, Glupczynski Y. 2006. Outbreak of infection by carbapenem-resistant *Acinetobacter baumannii* producing the carbapenemase OXA-58 in Belgium. J Clin Microbiol. 44:4189–4192.

Chen ZH, Liu WE, Zhang YL, Li YB, Jian ZJ, Deng HL, Zou MX, Liu YY. 2013. Molecular epidemiology of carbapenem-resistant *Acinetobacter* spp. from XiangYa Hospital, in Hunan Province, China. J Basic Microbiol. 53:121–127.

Mendes RE, Spanu T, Deshpande L, Castanheira M, Jones RN, Fadda G. 2009. Clonal dissemination of two clusters of *Acinetobacter baumannii* producing OXA-23 or OXA-58 in Rome, Italy. Clin Microbiol Infect. 15(6):588–592.

Yang SC, Chang WJ, Chang YH, Tsai YS, Yang TP, Juan CW, Shiau MY. 2010. Prevalence of antibiotics resistance and OXA carbapenemases genes in multidrug-resistant *Acinetobacter baumannii* isolates in central Taiwan. Eur J Clin Microbiol Infect Dis. 29:601–604.

Zong Z, Lü X, Valenzuela JK, Partridge SR, Iredell J. 2008. An outbreak of carbapenem-resistant *Acinetobacter baumannii* producing OXA-23 carbapenemase in western China. Int J Antimicrob Agents. 31:50–54.

Jiang MJ, Zhao SP, Li JM, Zhang FS. 2013. Molecular epidemiological study and detection of multi-drug resistant *Acinetobacter baumannii* related resistance genes. Afr J Microbiol Res. 7:5496–5502.

Jiang MJ, Zhang ZJ, Zhao SP. 2014. Epidemiological characteristics and drug resistance analysis of multidrug-resistant *Acinetobacter baumannii* in a China hospital at a certain time. Pol J Microbiol. 63:275–281.

Allen KD, Green HT. 1987. Hospital outbreak of multi-resistant *Acinetobacter anitratus*: an airborne mode of spread? J Hosp Infect. 9:110–119.

Obbard JP, Fang LS. 2003. Airborne concentrations of bacteria in a hospital environment in Singapore. Water Air Soil Pollut. 144:333–341.

Andersen AA. 1958. New sampler for the collection, sizing, and enumeration of viable airborne particles. J Bacteriol. 76:471–484.

Magiorakos AP, Srinivasan A, Carey RB, Carmeli Y, Falagas ME, Giske CG, Harbarth S, Hindler JF, Kahlmeter G, Olsson-Liljequist B, et al. 2012. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. Clin Microbiol Infect. 18(3):268–281.

Ribot EM, Fair MA, Gautom R, Cameron DN, Hunter SB, Swaminathan B, Barrett TJ. 2006. Standardization of pulsed-field gel electrophoresis protocols for the subtyping of *Escherichia coli* O157:H7, *Salmonella*, and *Shigella* for PulseNet. Foodborne Pathog Dis. 3(1):59–67.

Bartual SG, Seifert H, Hippler C, Luzon MAD, Wisplinghoff H, Rodríguez-Valera F. 2005. Development of a multilocus sequence typing scheme for characterization of clinical isolates of *Acinetobacter baumannii*. J Clin Microbiol. 43:4382–4390.

Shen J, Zhu D, Wang M. 2008. The relationship between acquired carbapenemases and resistance of gram-negative bacilli. Chin J Lab Med. 31:408–414.

Yang Y, Ye H, Zhou X. 2011. Detection of New Delhi metallo-β-lactamase I gene in *Klebsiella ozaenae* and *Acinetobacter baumannii*. Chin J Lab Med. 32:1407–1409.

Obeidat N, Jawdat F, Al-Bakri AG, Shehabi AA. 2014. Major biologic characteristics of *Acinetobacter baumannii* isolates from hospital environmental and patients' respiratory tract sources. Am J Infect Control. 42:401–404.

Spellberg B, Bonomo RA. 2013. "Airborne Assault": A new dimension in *Acinetobacter baumannii* transmission. Crit Care Med. 41(8):2042–2044.

Munoz-Price LS, Fajardo-Aquino Y, Arheart KL, Cleary T, DePascale D, Pizano L, Namias N, Rivera JI, O'Hara JA, Doi Y. 2013. Aerosolization of *Acinetobacter baumannii* in a trauma ICU. Crit Care Med. 41(8):1915–1918.

Yakupogullari Y, Otlu B, Ersoy Y, Kuzucu C, Bayindir Y, Kayabas U, Togal T, Kizilkaya C. 2016. Is airborne transmission of *Acinetobacter baumannii* possible: A prospective molecular epidemiologic study in a tertiary care hospital. Am J Infect Control. 44: 1595–1599.

Salipante SJ, SenGupta DJ, Cummings LA, Land TA, Hoogestraat DR, Cookson BT. 2015. Application of whole-genome sequencing for bacterial strain typing in molecular epidemiology. J Clin Microbiol. 53:1072–1079.

Lee JY, Ko KS. 2012. Antimicrobial resistance and clones of *Acinetobacter* Species and *Pseudomonas aeruginosa*. J Bacteriol Virol. 42(1):1.

Le Hello S, Falcot V, Lacassin F, Baumann F, Nordmann P, Naas T. 2008. Molecular epidemiology of carbapenem-resistant *Acinetobacter baumannii* in New Caledonia. Clin Microbiol Infect. 14:977–981.

Mosqueda N, Espinal P, Cosgaya C, Viota S, Plasensia V, Alvarez-Lerma F, Montero M, Gomez J, Horcajada JP, Vila J, et al. 2013. Globally expanding carbapenemase finally appears in Spain: nosocomial outbreak of *Acinetobacter baumannii* producing plasmid-encoded OXA-23 in Barcelona, Spain. Antimicrob Agents Chemother. 57:5155–5157.

Runnegar N, Sidjabat H, Goh HMS, Nimmo GR, Schembri MA, Paterson DL. 2010. Molecular epidemiology of multidrug-resistant *Acinetobacter baumannii* in a single institution over a 10-year period. J Clin Microbiol. 48:4051–4056.

Liu LL, Ji SJ, Ruan Z, Fu Y, Fu YQ, Wang YF, Yu YS. 2015. Dissemination of bla_{OXA-23} in *Acinetobacter* spp. In China: main roles of conjugative plasmid pAZJ221 and Transposon Tn2009. Antimicrob Agents Chemother. 59:1998– 2005.

Ruan Z, Chen Y, Jiang Y, Zhou H, Zhou Z, Fu Y, Wang H, Wang Y, Yu Y. 2013. Wide distribution of CC92 carbapenem-resistant and OXA-23-producing *Acinetobacter baumannii* in multiple provinces of China. Int J Antimicrob Agents. 42:322–328.

Yokoyama K, Doi Y, Yamane K, Kurokawa H, Shibata N, Shibayama K, Yagi T, Kato T, Arakawa Y. 2003. Acquisition of 16S rRNA methylase gene in *Pseudomonas aeruginosa*. Lancet. 362: 1888–1893.

Polish Journal of Microbiology
2018, Vol. 67, No 3, 339–345
https://doi.org/10.21307/pjm-2018-041

ORIGINAL PAPER

Bacterial Microbiota and Fatty Acids in the Faeces of Overweight and Obese Children

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Submitted 28 December 2017, 28 February 2018, accepted 3 June 2018

Abstract

The growing number of children with overweight and obesity constitutes a major health problem of the modern world and it has been suggested that intestinal microbiota may influence energy intake from food. The objectives of this study were to determine quantity and proportions of dominant genera of *Bacteroides, Prevotella* (phylum *Bacteroidetes*); *Clostridium*, *Lactobacillus* (phylum *Firmicutes*) and *Bifidobacterium* (phylum *Actinobacteria*) in the intestines and to determine the content of short-chain fatty acids (SCFAs) and branched-chain fatty acids (BCFAs) in the stool of 20 obese children and 20 children with normal body weight. Strains classified as *Firmicutes* (*Clostridium* and *Lactobacillus*) predominated in stool microbiota of obese children, while those of *Bacteroidetes* (*Prevotella* and *Bacteroides*) were in minority ($p < 0.001$). Concentration of SCFAs in the stool of obese children was lower in comparison to the stool of normal weight children ($p = 0.04$). However, these differences were significant only in obese children, not in overweight children in comparison with the lean ones. Therefore, in our study obesity was associated with intestinal dysbiosis and a predominance of phylum *Firmicutes*. Secondly, stool of obese children contained lower amounts of SCFAs.

Key words: *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, BCFAs, obesity, SCFAs

Introduction

Obesity is considered a global epidemic (Report WHO, 2008; Report WHO, 2009). The causes of obesity are very complex, from bad habits such as intake of high amount of fats and simple sugars, via environmental conditions, stressogenic and genetic factors, as well as the influence of intestinal microbiota and short-chain fatty acids. The pathologically increased amount of fat causes a number of disorders in the proper functions of the systems, organs and tissues. Complications of the cardiovascular, respiratory, endocrine system and psychosocial problems proved to be particularly dangerous. Obesity is not only a medical problem, which directly contributes to 10–13% of premature deaths in Europe, but it is also associated with a significant economic burden because of the costs associated with obesity which in Europe range from 2% to 7%. A major problem is the phenomenon of transferring childhood obesity to adulthood (Maziak et al. 2007; Report WHO 2008; Report WHO 2009).

In 2004, Gordon hypothesized that intestinal microbiota is associated with body mass control of the host (Backhed et al. 2004). According to his assumptions, certain groups of bacteria are capable to absorb nutrients and energy more efficiently, and individuals, whose intestinal microbiota can rapidly metabolize nutrients, absorb more calories and increase body weight much easier; therefore, they are prone to obesity. It is believed that the specific relative ratio between *Bacteroidetes*

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and *Firmicutes*, which both constitute 90% of bacteria colonizing the intestines, is associated with obesity. The overgrowth of bacteria of phylum *Firmicutes* with simultaneous reduction of bacteria from phylum *Bacteroidetes* was observed in the intestines of both obese mice and humans (Backhed et al. 2004; Ley et al. 2006; Sanz et al. 2008; De Filippo et al. 2010; Diamant et al. 2011; Everard et al. 2013, Canfora et al. 2015; Lu et al. 2016; Raza et al. 2017).

The specific ratio of *Bacteroidetes* to *Firmicutes* is important for a healthy body weight; and beside this, the gut microbiota have many beneficial functions, such as: the influence on immunity, stimulation of the microvilli development, fermentation of dietary fiber and prebiotics: short-chain fatty acids (SCFAs) (butyric, propionic and acetic acids) and lactic acid that are very beneficial to humans (Neish 2002; Stewart et al. 2004; Cani et al. 2008; Cani et al. 2009; Walker and Lawley 2013). This observation has recently been questioned since different studies failed to confirm differences in the *Bacteroidetes*/*Firmicutes* ratio between lean and obese humans (Mondot and Lepage 2016; Lin et al. 2016; Bouter et al. 2018). Metabolism of glucose is regulated by the gut microbiota. It was shown that the high number of bacteria belonging to the *Bacteroidetes* phylum significantly affects glucose intolerance, which was caused by the consumption of a high-fat diet. However, the high *Bacteroidetes* content had little impact on mouse obesity (Rabot et al. 2016). The individual's microbiota composition may cause phenotypic variation correlated to mice feeding with high-fat diet (Rabot et al. 2016).

SCFAs have many functions; among them, propionic acid has a positive effect on the growth of hepatocytes and acetic acid on the development of peripheral tissues. Butyric acid stimulates the growth of intestinal epithelial tissue, nourishes intestinal cells and affects their proper maturation and differentiation. SCFAs regulate also glucose and lipid metabolism, stimulate proliferation and differentiation of intestinal enterocytes, contribute to decrease in the pH of intestinal content and promote the absorption of minerals by increasing their solubility (Blaut and Clavel 2007; Lin et al. 2012).

The objective of this study was to determine the prevalence and proportions of dominant bacteria of genera *Bacteroides*, *Prevotella* (phylum *Bacteroidetes*); *Clostridium*, *Lactobacillus* (phylum *Firmicutes*) and *Bifidobacterium* (phylum *Actinobacteria*) in the intestines as well as to determine the content of short-chain fatty acids (acetic, butyric, formic, propionic, valeric) and branched-chain fatty acids (isovaleric and isobutyric) in the stool of 20 obese children and 20 children with normal body weight.

Experimental

Materials and methods

Biological material. The stool of 20 children: 10 overweights (BMI 25,68–29,48) and 10 obese children (BMI 31,71–41,18) aged between 6 and 15 years, and stool of 20 lean children (BMI 18,5–22,38) in the same age range was collected. As many as 55% of overweight and obese children lived in the city, while others (45%) were from rural areas. Children with normal body weight lived mostly in the city (70%) while 30% were from rural areas. In the group of overweight and obese children, girls and boys accounted for 60% and 40%, respectively. In the group of children with normal body weight, girls and boys constituted 65% and 35%, respectively. In the close family of overweight and obese children, one could report overweight or obesity in over 95% cases; while in children from family where normal body weight was reported – only 15% of family members were overweight or obese. Definitions of overweight and obesity were based on definition of the International Obesity Task Force (IOTF) criteria developed by Cole et al. (2012). Immediately after the samples were taken into sterile containers, the faeces were frozen and transported on the same day for further analysis.

Determination of the number of bacteria. The number of bacteria was determined with fluorescence *in situ* hybridization (FISH). Hybridization procedure was prepared based on the method described by Barczyńska et al. (2017). Hybridization was conducted in a humid chamber at a temperature and time specific to the oligonucleotide probes applied. Table I shows the sequences of probes and hybridization conditions used

Table I
The sequence of oligonucleotide probes and hybridization conditions used in FISH procedure for the identification of the bacteria present in children faeces.

Probe	Identified microorganisms	Sequence (5'→3')	Fluorescent label	Temp [°C]	Time [h]
Lab 158	*Lactobacillus-Enterococcus*	GGT ATT AGC A(T?C)CTGT TTC CA	5'Fluo	46	24
Bif 164	*Bifidobacterium* spp.	CAT CCG GCA TTA CCA CCC	5'Cy3	58	18
Bac 303	*Bacteroides-Prevotella*	CCA ATG TGG GGG ACC TT	5'Cy3	55	3
Erec 484	*Clostridium coccoides*	GCT TCT TAG TCA GGT ACC G	5'Cy3	57	16
Prov	*Prevotella*	ATCTTGAGTGAGTTCGATGTTGG	5'Fluo	57	18

for the identification of bacteria by FISH. Microscopic observations were performed using Eclipse E-400 fluorescence microscope (Nikon, Japan), combined with COHU 4910 camera (Cohu Inc., USA) and coupled with a computer. Enumeration of microbial cells was performed using NIS Elements BR version 3.2 computer program (Nikon, Japan).

Isolation, amplification, RFLP and sequencing of DNA. Isolation of bacteria genomic DNA was performed using kit Genomic Mini (A&A Biotechnology) according to the manufacturer's protocol. The isolated DNA was amplified by PCR using oligonucleotides specific to bacterial 16S RNA fragments. For sequencing purposes, the PCR products were purified using Exonuclease I (EURx) and Fast Polar-BAP kit (EURx). For restriction enzyme length polymorphism analysis (RFLP), the PCR products were digested using Taq I restriction enzyme (Thermo Scientific) and compared to control DNA mass marker. Automated sequencing of purified DNA fragments was performed in the Laboratory of DNA Sequencing and Oligonucleotides Synthesis at the Institute of Biochemistry and Biophysics of Polish Academy of Sciences at Warsaw using 3730 XL DNA Analyzer (Applied Biosystem). Sequence data of the selected isolates were analyzed and compared to sequences of reference strains obtained from Gen Bank NCBI base using BLASTN 2.2.32+ program.

Analysis of the content of SCFA and BCFA acids by high performance liquid chromatography, HPLC. Determination of the concentrations of lactic acid, SCFAs (acetic, propionic, butyric, formic, and valeric acids) and BCFAs (isobutyric and isovaleric acids) was conducted using high performance liquid chromatography (HPLC) with Surveyor liquid chromatograph (Thermo Scientific). Aminex HPX-87H (300 × 7.8 mm) (Bio-Rad Aminex®) column filled with styrene-divinylbenzene sulfonated copolymer bed were used. The following parameters were used in the analyses: Aminex HPX-87H column, mobile phase 0.005 M H_2SO_4, UV detector at a wavelength of 210 nm, rheodyne type injection valve, injection of sample – 10 µl, analysis temperature –60°C, flow rate 0.6 µl min^{-1}, analysis time of a single sample 35 min. HPLC analysis was performed for the samples with known concentrations of appropriate acids, as follows: 0, 0.125, 0.25, 0.50, 0.75 and 1% to plot calibration curves, i.e., the concentration of the acid in the function of surface area of the peak shown in the chromatogram (area). The equations developed were based on calibration curves and enabled calculation of the concentration of short-chain fatty acids in the analyzed stool samples.

Statistical analysis. The results were evaluated with W-Shapiro Wilk test in order to assess the normality of the distribution of the results. Due to the deviation from the normal distribution, further analyses were performed with use of U Mann-Whitney test. The data obtained from the questionnaire were analysed using the χ^2 test. Statistical significance was established at $p < 0.05$. The statistical analysis was performed using STATISTICA 10.0 software (StatSoft, Inc.).

Results

Determination of the number of bacteria of the main genera from overweight, obese and normal weight children. It was found that in the stool of overweight and obese children, among of the five bacteria genera tested, those classified as *Clostridium* predominated and their number was from 7.21 to 8.96 $\log_{10}$ cells g^{-1}, average 8.03 $\log_{10}$ cells g^{-1}. In the stool of children with normal weight, the average amount of these bacteria was lower by about 14% ($p < 0.01$), and their number was from 5.42 to 7.92 $\log_{10}$ cells g^{-1}. In the stool of children with normal body weight the dominant genera were *Bacteroides* (their number was from 6.58 to 10.06 $\log_{10}$ cells g^{-1}, average 8.57 $\log_{10}$ cells g^{-1}) and *Bifidobacterium* (their number was from 6.78 to 9.52 $\log_{10}$ cells g^{-1}, average was 9.07 $\log_{10}$ cells g^{-1}). In overweight and obese children, the number of *Bacteroides* was lower by about 20% ($p < 0.01$) (6.67 to 8.0 $\log_{10}$ cells g^{-1}), and *Bifidobacterium* by about 18% ($p = 0.036$) (6.51 to 8.48 $\log_{10}$ cells g^{-1}). The number of *Prevotella* was higher on average by 30% ($p < 0.01$) in children with normal body weight (the number was from 3.25 to 8.98 $\log_{10}$ cells g^{-1}) compared to children with obesity (the number was from 3.19 to 5.18 $\log_{10}$ cells g^{-1}). The average number of *Lactobacillus* strains in the stool of overweight, obese and normal weight children was similar and accounted for 7.81 $\log_{10}$ cells g^{-1} (6.61 to 9.52 $\log_{10}$ cells g^{-1}), and 7.77 $\log_{10}$ cells g^{-1} (7.16 to 8.65 $\log_{10}$ cells g^{-1}), respectively ($p = 0.913$) (Fig. 1).

Systematizing the strains tested to phyla, it was found that in the stool of overweight and obese children, bacteria of phylum *Firmicutes* (*Lactobacillus* and *Clostridium* genera) accounted for the majority of the bacteria tested, on average 45.9%. In the faeces of overweight and obese children, the bacteria belonging to genera *Lactobacillus and Clostridium* was similar and accounted for 23% of bacteria population from the five tested bacteria genera. However, bacteria belonging to phylum *Bacteroidetes* (*Prevotella and Bacteroides* genera) accounted on average for 32.4% of bacteria. Within this phylum *Bacteroides* dominated (19.89%), while *Prevotella* was in the minority (12.5%). *Bifidobacterium* (phylum *Actinobacteria*) accounted for 21.7% of the bacteria. In the stool of children with normal weight, the relative proportion of the major types of bacteria was different in comparison to overweight and obese children. The number of bacteria classified to

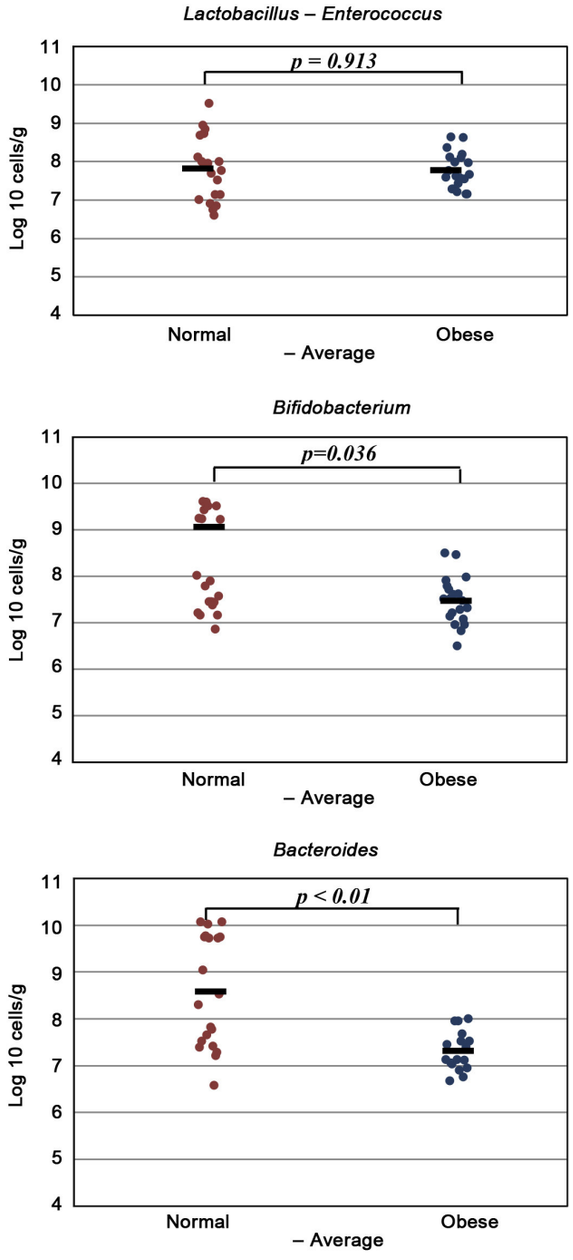

Fig. 1. The number of bacteria isolated from stool of overweight, obese and normal weight children

Firmicutes phylum (*Lactobacillus* and *Clostridium* genera) was lower when compared to the previous group and accounted for on average 39.1% of the bacterial population investigated. Bacteria from genera *Clostridium* and *Lactobacillus* accounted for 18.4% and 20.7% of bacterial population from the five bacteria genera tested. The 39% of bacteria was classified to *Bacteroidetes* phylum, 22.7% to *Prevotella,* and 16.3% to *Bacteroides.* The *Bifidobacterium* strains (*Actinobacteria*) represented 21.9% of total bacterial population in the stool of overweight, obese and normal weight children (Fig. 2a, 2b).

Among the group of obese children, three of them have a high BMI equal to 40.1. In this group of children, a high abundance of *Firmicutes* (53.8%, among them *Lactobacillus* 26.4% and *Clostridium* 27.4%) and low of *Bacteroidetes* (25%, among them *Prevotella* 11.3% and *Bacteroides* 13.6%) as well as *Bifidobacteria* (21%) when compared to slim children. However, the number of individuals in this group is too small to perform a statistical analysis (Fig. 2c).

RFLP and sequencing analysis. RFLP analysis of the 16S rRNA amplicons digested with Taq I restriction enzyme showed that the bacteria investigated in this study belong to various groups, as: *Bacteroides, Bifidobacterium, Clostridium, Lactobacillus and Prevotella.* Bacteria classified to one strain had identical RFLP pattern. It was confirmed by sequencing analysis of bacterial 16S rRNA amplicons, whose sequences were compared to nucleotide sequences of the reference bacterial strains obtained from National Center for Biotechnology Information (NCBI).

SCFA and BCFA in the stool of overweight, obese and normal weight children. In the stool of overweight, obese and lean children, lactic acid dominated. The concentration of lactic acid in the stool of overweight and obese children ranged from 0.017 to 4.351 mg g^{-1} of stool (1.35 ± 0.19 mg g^{-1} of stool), and in children with normal body weight it ranged from 0.093 to 4.909 mg g^{-1} of stool (2.24 ± 0.32 mg g^{-1} of stool; $p = 0{,}014$) (Table II). The concentration of SCFAs in the stool of overweight and obese children ranged from 0.342 to 7.521 mg g^{-1} of stool (3.59 ± 0.49 mg g^{-1} of stool), and BCFAs from 0.022 to 0.896 mg g^{-1} of stool (0.36 ± 0.05 mg g^{-1} of stool) (Table II). The concentration of SCFAs in the stool of children with normal body weight was higher, ranged from 0.424 to 10.18 mg g^{-1} of stool (5.44 ± 0.76 mg g^{-1} of stool, $p = 0{,}040$), and the BCFAs concentration fluctuated from 0.001 to 1.151 mg g^{-1} of stool (0.44 ± 0.06 mg g^{-1} of stool, $p = 0{,}741$). Concentrations of formic, valeric and butyric acids in stool did not differ between groups. Similarly, concentrations of acetic and propionic acids were similar in both groups.

In the stool of overweight and obese children isovaleric acid occurred at the highest concentration among BCFAs. Its concentration was equal to 0.20 mg g^{-1} of stool, while the average isobutyric acid concentration was equal to 0.16 mg g^{-1} of stool (Table II). In the stool of children with normal body weight the concentration of BCFA isovaleric and isobutyric acids was similar and was equal to 0.20 mg g^{-1} of stool ($p = 0.913$) and 0.23 mg g^{-1} of stool ($p = 0.640$), respectively.

The results presented in Table II showed that there are significant differences in intestinal microbiota between obese and lean children. Secondly, overweight and obese children have significantly disturbed composition of fatty acids in stool.

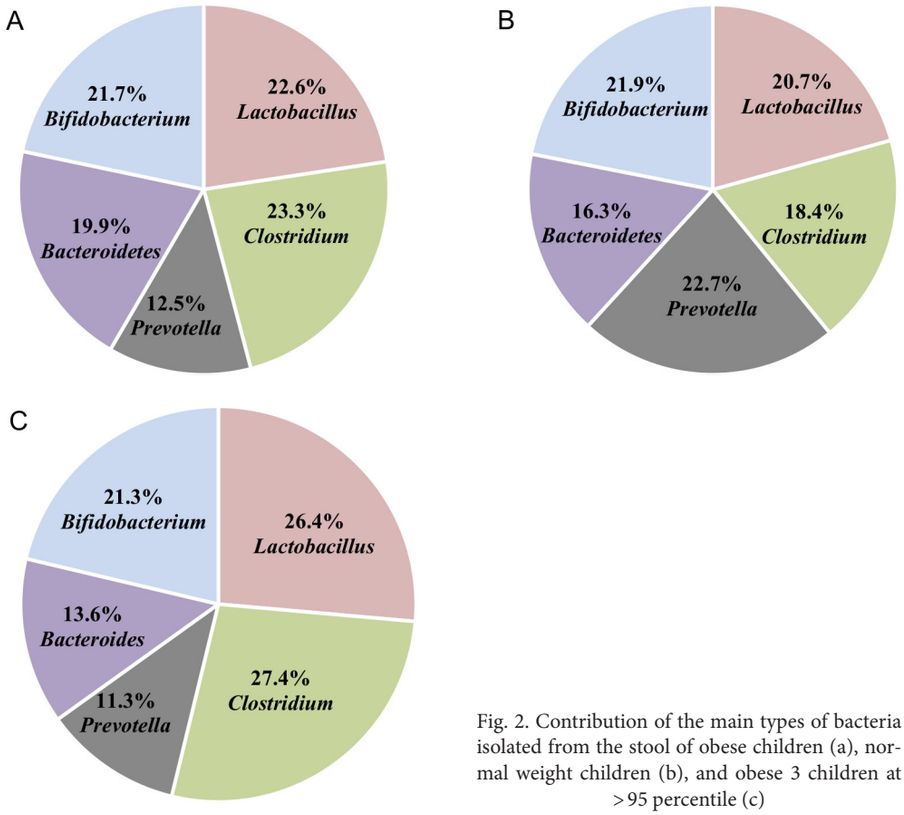

Fig. 2. Contribution of the main types of bacteria isolated from the stool of obese children (a), normal weight children (b), and obese 3 children at >95 percentile (c)

Discussion

The development of obesity is hypothesized to be closely related to changes in intestinal microbiota and the current results of both experimental and clinical studies indicate significant differences in microbiota and bacterial gut metagenomes of obese adults and children compared with lean individuals (Backhed et al. 2004; Kimm et al. 2005; Ley et al. 2006; Sanz et al. 2008; De Filippo et al. 2010; Shen et al. 20013). It was demonstrated that modifications of the intestinal microbiota towards the dominance of *Bacteroidetes* over *Firmicutes* correlated with decreased body weight in overweight and obese individuals (Backhed

Table II
SCFAs and BCFAs in the stool of overweight, obese and normal weight children.

Acid	Obese children			Children with normal weight			
	Acid concentration [mg g^{-1} stool]	Average [mg g^{-1} stool]	Median [mg g^{-1} stool]	Acid concentration [mg g^{-1} stool]	Average [mg g^{-1} stool]	Median [mg g^{-1} stool]	p
Lactic	0.017–4.351	1.35	1.126	0.093–4.909	2.24	1.804	0.014
Acetic	0.111–1.289	0.71	0.650	0.026–3.269	1.38	1.122	0.279
Propionic	0.085–1.232	0.67	0.594	0.050–2.128	1.08	0.985	0.354
Butyric	0.014–0.543	0.40	0.381	0.030–0.948	0.33	0.299	0.446
Formic	0.008–0.660	0.20	0.199	0.010–0.484	0.21	0.154	0.645
Valeric	0.012–0.412	0.26	0.254	0.063–0.472	0.20	0.187	0.164
Total SCFA	0.342–7.521	3.59	2.708	0.424–10.18	5.44	3.974	0.040
BCFA							
Isobutyric	0.048–0.341	0.16	0.100	0.020–0.545	0.23	0.200	0.640
Isovalerian	0.017–0.528	0.20	0.120	0.001–1.180	0.20	0.121	0.913
Total BCFA	0.022–0.896	0.36	0.255	0.001–1.151	0.44	0.421	0.741

Analysis was based on U Mann-Whitney test. Statistical significance was established at $p < 0.05$.

et al. 2004; Ley et al. 2006; Sanz et al. 2008; Turnbaugh et al. 2008; De Filippo et al. 2010; Diamant et al. 2011; Everard et al. 2013).

The composition of intestinal microbiota depends on many factors such as age, sex, diet, used medicines but also the region of the world and regional cuisine. The number of bacteria belonging to the main genera *Lactobacillus*, *Bifidobacterium*, *Prevotella*, *Bacteroides*, *Cliostridium* in the selected population of Polish children was determined for the first time in this work.

We found that the stool of normal weight Polish children contained higher number of bacteria (*Prevotella* and *Bacteroides*) classified to *Bacteroidetes* phylum than stool of overweight and obese children. However, when we analyzed obese children separately, we found clear dominance of bacteria of *Clostridium* and *Lactobacillus* genera classified to *Firmicutes* in comparison to *Bacteroidetes* (*Prevotella* and *Bacteroides*) observed only in three children, who have highest BMI and were eating food rich in animal fat.

A consequence of changes in the proportion of types of bacteria in the gut microbiota ecosystem is the modification of the proper production of SCFAs, which may also indirectly participate in the development of obesity (Canfora et al. 2015; Lu et al. 2016). The results of this study indicated that the concentration of SCFAs in the stool of overweight and obese children was lower in comparison to the stool of normal weight children. This phenomenon was also demonstrated for BCFAs.

In the stool of normal weight children, the decreasing concentrations of fatty acids (excluding lactic acid) were observed in the following order: acetic, propionic and butyric acid. In stool of overweight and obese children, these proportions were not so clear, and the propionic acid occurred at a similar concentration as acetic acid. The lower concentration of lactic acid in the stool of overweight and obese children then in the faeces of children with normal body mass correlated with the variable number of the main bacterial phyla. The higher concentration of lactic acid in the faeces of children with normal body weight was associated with a higher number of *Bifidobacteria* and comparable number of *Lactobacillus*, which is an effective producer of this acid. A similar relationship was demonstrated for propionic and acetic acid, the concentration of which was significantly higher in the faeces of children with normal body weight. This observation corresponded to the higher number of *Prevotella* and *Bacteroides* strains. On the other hand, the lower concentration of butyric acid was associated with a lower abundance of *Clostridium* in the faeces of children with normal body mass when compared to overweight and obese children. Pekmez et al. (2018) have shown that in faeces of obese children the higher concentrations of butyrate and propionate could be noticed when compared to lean children.

SCFAs are also ligands for the two G protein-coupled receptors – GPR41 and GPR43; however, they exhibit a different affinity for these receptors. GPR41 has a highest affinity toward propionic acid, followed by butyric and acetic acids, while the GPR43 receptor reacts similarly with each of these three acids (Arora et al. 2011). Binding of SCFAs, especially the propionic acid to GPR41 receptors, results in increased expression of leptin. Similarly, the binding of acetic acid to GPR43 receptors results in the increased secretion of leptin in the fatty tissue of the mice's mesentery and sodium propionate increases leptin secretion by 80% (Backhed et al. 2004; Arora et al. 2011; Nie et al. 2018).

The clarification of the mechanisms by which short-chain fatty acids (SCFAs) reduce body weight could help in the development of an strategy to effectively control the body weight. The body weight gain induced by a high-fat diet might be significantly inhibited by supplementation of diet with acetate, propionate, butyrate or their mixture. Significant changes in the expression of GPR43 and GPR41, characterized by increase of the adipose tissue mass and reduction in the colon, occurred due to the supplementation of SCFAs. Additionally, short-chain fatty acids influenced the change in microorganisms proportion in faeces by decreasing the abundance of *Firmicutes* and increasing the abundance of *Bacteroidetes* (Lu et al. 2016).

In the present study, we found that the concentration of propionic acid was lower in the stool of overweight and obese children than in the stool of normal weight children. The interpretation of this finding is difficult. The lower concentrations of propionic acid may be due its increased binding to G protein-coupled receptors as a ligand, and thus promoting inhibition of lipolysis and enhanced accumulation of lipids. On the other hand, lower concentrations of propionic acid may be associated with lower leptin secretion. Unfortunately, we did not measure leptin and other adipokines. The other interpretation of lower concentration of this SCFA in obese children is as a counterregulatory mechanism for increased caloric intake (Arora et al. 2011).

Our study has several limitations. First, we used conventional bacteriological methods and we do not assess bacterial metagenome. Second, our study group was small and we did not correlate our findings with metabolic abnormalities such as insulin resistance, dyslipidemia and immune activation. However, there are also few potential perspectives. It is interesting whether the interventions such as dietary modifications and/or increased physical activity may affect intestinal microbiome. Furthermore, it is important to analyze relation between intestinal microbiota and metabolic abnormalities typical for obesity and metabolic syndrome.

Concluding, we found that intestinal microbiome of overweight and obese children was significantly differ-

ent in comparison with lean children. It was accompanied by significant change in SCFAs and BCFAs content in stools of overweight and obese children. This research has been carried out on a selected population of Polish children using conventional microbiological methods. The recent scientific reports indicate that the role of intestinal microbiota in the pathogenesis of obesity is ambiguous and therefore it is justified to repeat the study on metabolome and metagenome of faecal samples from Polish children.

Acknowledgements

The study was supported by a grant from the National Science Centre number DEC-2011/03/D/NZ9/03601.

Literature

Arora T, Sharma R, Frost G. 2011. Propionate. Anti-obesity and satiety factor? Appetite. 56:511–515.

Backhed F, Ding H, Wang T, Hooper LV, Koh GY, Nagy A, Semenkovich CF, Gordon JI. 2004. The gut microbiota as an environmental factor that regulates fat storage. Proc Natl Acad Sci. 10:15718–15723.

Barczynska R, Jurgoński A, Slizewska K, Juśkiewicz J, Kapusniak J. 2017. Effects of potato dextrin on the composition and metabolism of the gut microbiota in rats fed standard and high-fat diets. J Funct Foods. 34:398–407.

Blaut M, Clavel T. 2007. Metabolic diversity of the intestinal microbiota: implications for health and disease. J Nutr. 137:751–755.

Bouter K, van Raalte DH, Groen AK, Nieuwdorp M. 2018. Role of the gut microbiome in the pathogenesis of obesity and obesity-related metabolic dysfunction. Gastroenterol. 152(7):1671–1678.

Canfora EE, Jocken JW, Blaak EE. 2015. Short-chain fatty acids in control of body weight and insulin sensitivity. Nat Rev Endocrinol. 11(10):577–591.

Cani PD, Bibiloni R, Knauf C, Waget A, Neyrinck AM, Delzenne NM, Burcelin R. 2008. Changes in gut microbiota control metabolic endotoxemia-induced inflammation in high-fat diet-induced obesity and diabetes in mice. Diabetes. 57:1470–1481.

Cani PD, Possemiers S, Van de WT, Guiot Y, Everard A, Rottier O, Geurts L, Naslain D, Neyrinck AM, Lambert DM, et al. 2009. Changes in gut microbiota control inflammation in obese mice through a mechanism involving GLP-2-driven improvement of gut permeability. Gut. 58:1091–1103.

Cole TJ, Lobstein T. 2012. Extended international (IOTF) body mass index cut-offs for thinness, overweight and obesity. Pediatr Obes. 7(4):284–294.

De Filippo C, Cavalieri D, Di Paola M, Ramazzotti M, Poullet JB, Massart S, Collini S, Pieraccini G, Lionetti P. 2010. Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rual Africa. Proc Natl Acad Sci. 107:14694–14696.

Diamant M, Blaak EE, de Vos WM. 2011. Do nutrient-gut-microbiota interaction play a role in human obesity, insulin resistance and type 2 diabetes? Obes Rev. 12:272–281.

Everard A, Lazarevic V, Derrien M, Girard M, Muccioli GM, Neyrinck AM, Possemiers S, Van Holle A, François P, de Vos WM, et al. 2011. Responses of gut microbiota and glucose and lipid metabolism to prebiotics in genetic obese and diet-induced leptin-resistant mice. Diabetes 60:2775–2786.

Everard A, Cani PD. 2013. Diabetes, obesity and gut mikrobiota. Best Pract Res Clin Gastroenterol. 27:1–3.

Kimm SY, Glynn NW, Obarzanek E, Kriska AM, Daniels SR, Barton BA, Liu K. 2005. Relation between the changes in physical activity and body-mass index during adolescence: a multicentre longitudinal study. Lancet. 366(9482):301–307.

Ley RE, Turnbaugh P, Klein S, Gordon JI. 2006. Human gut microbes associated with obesity. Nature. 444:1022–1023.

Lin HV, Frassetto A, Kowalik EJ, Nawrocki AR, Lu MM, Kosinski JR, Hubert JA, Szeto D, Yao X, Forrest G, Marsh DJ. 2012. Butyrate and propionate protect against diet-induced obesity and regulate gut hormones via free fatty acid receptor 3-independent mechanisms. Plos ONE. 7:35240.

Lin H, An Y, Hao F, Wang Y, Tang H. 2016. Correlations of fecal metabonomic and microbiomic changes induced by high-fat diet in the pre-obesity state. Sci Rep. 6:21618.

Lu Y, Fan C, Li P, Lu Y, Chang X, Qi K. 2016. Short chain fatty acids prevent high-fat-diet-induced obesity in mice by regulating G protein-coupled receptors and gut microbiota. Sci Rep. 6:37589.

Maziak W, Ward KD, Stockton MB. 2007. Childhood obesity: are we missing the big picture? Obesity Rev. 9(1):35–42.

Mondot S, Lepage P. 2016. The human gut microbiome and its dysfunctions through the meta-omics prism. Ann N Y Acad Sci. 1372:9–19.

Neish AS. 2002. The gut microflora and intestinal epithelial cells: a continuing dialogue. Microbes Infect. 4:309–317.

Nie Y, Luo F, Lin Q. 2018. Dietary nutrition and gut microflora: A promising target for treating diseases. Trends Food Sci Technol. 75:72–80.

Pekmez CT, Dragsted LO, Brahe LK. 2018. Gut microbiota alterations and dietary modulation in childhood malnutrition – The role of short chain fatty acids. Clin Nutr. 1–16.

Rabot S, Membrez M, Blancher F, Berger B, Moine D, Krause L, Bibiloni R, Bruneau A, Gérard P, Siddharth J, et al. 2016. High fat diet drives obesity regardless the composition of gut microbiota in mice. Sci Rep. 6:32484.

Raza GS, Putaala H, Hibberd AA, Alhoniemi E, Tiihonen K, Mäkela KA, Herzig KH. 2017. Polydextrose changes the gut microbiome and attenuates fasting triglyceride and cholesterol levels in Western diet fed mice. Sci Rep. 7:5294.

Report WHO. 2008. Waist circumference and waist-hip ratio report of a WHO expert consultation. Geneva (Switzerland): World Health Organization.

Report WHO. 2009. Population-based prevention strategies for childhood obesity: report of a WHO forum and technical meeting. Geneva (Switzerland): World Health Organization.

Sanz Y, Santacruz A, De Palma G. 2008. Insight into the roles of gut microbes in obesity. Interdiscip Perspect Infect Dis. 2008:1–9.

Shen J, Obin MS, Zhao L. 2013. The gut microbiota, obesity and insulin resistance. Mol Aspects Med. 34:39–58.

Stewart CS, Duncan SH, Cave DR. 2004. Oxalobacter formigenes and its role in oxalate metabolism in the human gut. FEMS Microbiol Lett. 230:1–7.

Turnbaugh PJ, Bäckhed F, Fulton L, Gordon JI. 2008. Diet-induced obesity is linked to marked but reversible alterations in the mouse distal gut microbiome. Cell Host Microbe. 3:213–223.

Walker AW, Lawley TD. 2013. Therapeutic modulation of intestinal dysbiosis. Pharmacol Res. 69:75–86.

Polish Journal of Microbiology
2018, Vol. 67, No 3, 347–353
https://doi.org/10.21307/pjm-2018-042

ORIGINAL PAPER

RNA Quality Control Using External Standard RNA

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Submitted 19 March 2018, revised 6 May 2018, accepted 14 June 2018

Abstract

In this paper, we propose a new evaluation method using external standard RNA for quality control of the extracted RNA. RNA Integrity Number and UV absorption are generally used as a basis for RNA quality control; however, these methods do not always reflect the quality of mRNA. While standard RNA is supposedly designed on the basis of mRNA, it has the potential to be used to evaluate the quality of the mRNA. In this study, we took into consideration the three essential factors, viz., yield of mRNA, inhibition to DNA polymerase, and degradation of mRNA for determining the RNA quality using standard RNA. It would be possible to know yield of mRNA and inhibition of the enzyme reaction by adding standard RNA before RNA extraction and looking at standard RNA loss. Degradation was evaluated by comparing the differences in the 3' and 5' regions of the RNA. In our study, it was demonstrated that in the crude extract of *Saccharomyces cerevisiae*, degradation was comparatively higher at the 3' end of RNA than at the 5' end. Hence, the degree of RNA degradation can be evaluated by comparing the ratio of degradation from the 3' and 5' end.

Key words: RNA degradation, RNA quality control, Standard RNA

Introduction

Expression levels of functional mRNA denote the essential functions that are required to retain life under various conditions. Thus, the degree of mRNA expression is widely employed to understand the cellular conditions (Wang et al. 2009; Qin et al. 2016). Therefore, northern blotting, RT-qPCR, and DNA microarray were employed to quantify mRNA. Furthermore, RNA sequencing using a next-generation DNA sequencer and Digital RT-PCR is essential to study "omics". Technologies focusing on mRNA are steadily developing and offer higher sensitivity, whereas techniques for determining the quality of extracted mRNA are still not at par. To analyze functional mRNA, it is essential to establish a more precise RNA quality control method (Kashofer et al. 2013; Li et al. 2015).

In this study, we validated a new RNA quality control method using an external standard. We used the RNA Solutions by Qualitative Analysis (AIST, Japan) as the external standard RNA. Standard RNAs have the potential to be used to evaluate mRNA directly because standard RNAs are designed based on human mRNA (Tong et al. 2006). Standard RNA has already been used to evaluate RNA yield (Takahashi et al. 2013). However, in this study we focused not only on yield but also RNA degradation. Standard RNAs are available in five different types, viz., three each of 533-nt and two each of 1033-nt, and are designed in a way that they share low homology sequences with natural sequences. Furthermore, they have the potential to be used to directly evaluate certain factors simultaneously. For this study, we evaluated the yield of mRNA from cells, inhibition by contaminants, i.e. unknown cellular component such as DNA polymerase, and degradation of mRNA by using standard RNA.

RNA yield is a factor denoting the quality, but it is usually ignored as a base for evaluation of quality control. Low yield of RNA suggest that the extraction procedure is not appropriate or that cellular disintegration causes RNA damage (Kashofer et al. 2013). We evaluated the final yield of total RNA and not the efficiency of RNA extraction. Comparison of RNA expression from different samples with different efficiencies of RNA yield may affect the final outcome. Therefore, we evaluated the final yield of RNA and the efficiency

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of yield during RNA extraction. In this study, RNA was extracted from *Saccharomyces cerevisiae* and *Escherichia coli* by the hot phenol method (Sambrook et al. 1989). Standard RNA was added to both RNA extraction procedures and the efficiency of yield was evaluated for RNA extraction by measuring the amount of standard RNA in the RNA-extracted solution.

The prepared RNA samples are not always free of contaminants since they can be contaminated with proteins and polysaccharides; these contaminants can lead to unwanted enzymatic reactions that inhibit RNA extraction and, hence, denote false positive results (Pionzio and McCord 2014). A260/A230 and A260/A280 ratios are generally used as a base for determination of RNA quality (Sambrook et al. 1989; Manchester 1996). Absorbance at 230 nm usually denotes contamination by organic solvents or TE. Absorbance at 260 nm denotes mass of nucleic acids; RNA and DNA. Absorbance at 280 nm denotes contamination by proteins or DDT. Contamination, measured by the A260/A280 ratio, is considered to inhibit reaction of enzymes (e.g., reverse transcriptase and DNA polymerase). A260/A280 and A260/A230 ratios are widely accepted for evaluating the contamination in the prepared RNA samples. However, this ratio does not directly evaluate inhibition. Thus, standard RNA can be used to evaluate the degree of unknown contamination that may inhibit enzyme reaction. In this study, we employed real-time qPCR to quantify the final value of standard RNA and to determine the quantity of inhibition in RNA-extraction solutions of *S. cerevisiae* and *E. coli*.

Evaluation of mRNA degradation is not easily accepted by organizations that are responsible to record the values obtained from all prepared mRNA samples. RNA Integrity Number (RIN) value and 18S/28S ribosomal ratio are the most used parameters for determining mRNA quality control (Imbeaud et al. 2005). This method considers two kinds of rRNA as internal standards. The extracted total RNA is subjected to capillary gel electrophoresis, and an electropherogram is constructed from the fluorescence for determining retention time. By comparing two peaks of rRNA and with the ideal peak, RNA quality is evaluated. However, because this method evaluates rRNA that comprises a major portion of the total RNA, it does not always reflect the quality of mRNA, which comprises a small portion of the total RNA (Feng et al. 2015). While standard RNA is designed on the basis of mRNA, it has the potential to evaluate mRNA quality. In this study, we evaluated mRNA degradation using standard RNA. We also validated the difference in degradation of RNA structure from the 3' and 5' ends.

This study, hence, helps in establishing a method using external standard RNA that can be used to directly evaluate the extracted mRNA quality.

Experimental

Materials and Methods

Standard RNAs. We used RNA solutions for qualitative analysis (AIST, Japan) as external standard RNA. This standard RNA is available in five different types: 500-A, 500-B, and 500-C are of 533 nt each and 1000-A and 1000-B are of 1033 nt each. Concentration of the solution was 33.4, 32.2, 32.1, 68.2, and 64.1 ng/µl, respectively. 10^{-3} diluted standard RNAs were used in this experiment. Standard RNA 500-B has low complementary sequences and tends to form a single strand. Standard RNA 500-C has high complementary sequences and tends to form double strands. The secondary structures of both the standard RNA were predicted using the software "CentroidFold" (http://www.ncrna.org/centroidfold).

Strain and growth conditions. We used *E. coli* strain JM109 (TakaraBio, Japan) [*recA1, endA1, gyrA96, thi, hsdR17* (*rK– mK+*), *e14–* (*mcrA–*), *supE44, relA1*, *Δ*(*lac–proAB*)/*F"*(*traD36, proAB+, lac Iq, lacZΔM15*)] and *S. cerevisiae* strain S288C (NBRC 1136, Japan) [*MATα SUC2 mal mel gal2 CUP1* (*cir+*)] for RNA extraction. *E. coli* was cultured in lysogeny broth (LB), comprising of 1% tryptone, 0.5% yeast extract, and 1% NaCl, and incubated at 37°C in an incubator-shaker with the rotation speed set at 120 rpm. *S. cerevisiae* was cultured in yeast peptone dextrose (YPD) broth comprising of 2% peptone, 1% yeast extract, and 2% glucose, and incubated at 30°C in an incubator-shaker with the rotation speed set as 120 rpm. *E. coli* and *S. cerevisiae* were isolated from the 1 ml culture suspensions by centrifugation at 15 100 × g at 4°C for 1 min when the OD of the broth at 600 nm was 0.6, and the supernatant was discarded. *E. coli* and *S. cerevisiae* cells were washed by distilled water and collected. These samples were then used for RNA extraction.

RNA extraction by hot phenol method and evaluation of RNA yield. *E. coli* and *S. cerevisiae* were suspended in sodium acetate buffer (50 mM sodium acetate, 10 mM EDTA, pH 5.0). To prepare suspensions, 10 µl of 10^{-3} dilution of standard RNA 1000-A, 12.5 µl of 10% sodium dodecyl sulfate (SDS), and 300 µl of phenol were added, and the suspensions were incubated at 65°C for 5 min. The suspensions were then conjugated for 10 sec and incubated at 65°C for 10 sec; this step was repeated 10 times. Next, suspensions were centrifuged at 15 100 × g, at 25°C for 5 min. Supernatants were transferred to new tubes, and 10 µl of 10^{-3} dilution of standard RNA 1000-B was added. To these supernatant mixtures, 300 µl of chloroform was added, and the samples were conjugated and centrifuged at 15 100 × g at 25°C for 5 min. Then, 100 µl of supernatants were transferred to new tubes; 10 µl

of 10^{-3} dilution of standard RNA 500-A, 11 µl of 3 M sodium acetate, and 287 µl of 99.5% ethanol were added to the suspension. Suspensions were then incubated at −20°C for 4 h, centrifuged at 17,400 × g at 4°C for 30 min, and then supernatants were discarded. Next, 500 µl of cold 70% ethanol was added to the precipitate and centrifuged at 17,400 × g at 4°C for 1 min. The supernatants were separated. Precipitates were dissolved in 100 µl of nuclease-free water. These suspensions were designated as "RNA extraction."

Real-time qPCR for evaluation of RNA yield and inhibition of enzymes. To evaluate yield of RNA, each standard RNA in all RNA extractions was quantified by one step real-time qPCR using One Step SYBR® PrimeScript™ RT-PCR Kit II Perfect Real Time (Takara-Bio, Japan). Table I lists the primers used for real-time qPCR, which targets standard RNA 1000-A, 1000-B and 500-A. To evaluate inhibition of the enzyme, we also quantified samples which same amount of standard RNAs was added to RNA extraction.

Calculation of the inhibitory effect. To evaluate the inhibitory effect caused by contaminants, we re-added standard RNA at the same concentrations to the extracted RNA solution. Concentration of RNA in this sample (A) and in the extracted RNA solution (B) were measured by RT-qPCR, and the differences between both values were calculated. These differences denoted the concentration of standard RNA to be re-added to the extracted RNA solution to quantify results, and we could determine the inhibitory effect that affected the yield of mRNA in the RNA-extracted solution. Inhibitory effect was calculated by the following formula:

Inhibitory effect $(\%) = 100 - [(A - B) \div C] \times 100$, where A denotes the concentration of standard RNA in the RNA-extracted solution in RT-qPCR, B denotes the concentration of standard RNA in the RNA-extracted solution, and C denotes the concentration of the added standard RNA.

Preparation of *E. coli* and *S. cerevisiae* crude extract. *E. coli* was isolated from the 1-ml culture suspension by centrifugation at 15 100 × g at 4°C for 1 min when the OD of the broth at 600 nm was 0.6. Then, 300 µl of PBS was added, and the suspension was subjected to sonication. The sonicated suspension was centrifuged at 15 100 × g at 4°C for 10 min, and supernatant was collected. The collected supernatant was diluted to the concentration of 10^{-1}, and the concentration of protein was calculated to be equal to 0.4 mg/ml. The protein concentration was measured by ultraviolet absorption spectrometry.

S. cerevisiae was isolated from the 10-ml culture suspension by centrifugation at 2 430 × g at 4°C for 10 min when the OD of the broth at 600 nm was 0.6. Next, 300 µl of PBS, acid-washed glass beads in the size range of 425–600 µm (Sigma, USA) were added to the suspension. The suspension was conjugated for 10 sec and incubated on ice for 10 sec; this process was repeated 10 times. The suspension was centrifuged at 15 100 × g at 4°C for 5 min, and supernatant was collected. The protein concentration was calculated to be equal to 36.7 mg/ml. These conditions were selected after several pre-experiments..

RNA degradation. Five microliters *E. coli* and *S. cerevisiae* crude extract was added to 20 µl of 10^{-3} dilutions of standard RNA 500-B and 500-C, respectively. Standard RNA-added *E. coli* crude extract was degraded for 0, 7.5, 15, 30, 60, and 120 min at 37°C. Standard RNA-added *S. cerevisiae* crude extract was degraded for 0, 10, 20, 60, and 240 min at 30°C. After degradation, 10 µl of 10^{-3} dilution of standard RNA 500-A was added to the solution, and the solution was purified by RNaeasy (Promega, USA). Standard RNAs were eluted by 50 µl of nuclease-free water.

Real time qPCR for evaluation of RNA degradation. Standard RNAs that were degraded by *E. coli* or *S. cerevisiae* crude extracts were transcribed using ReverTra Ace® qPCR RT Master Mix (Toyobo, Japan). All cDNAs were quantified using Power SYBR® Green PCR Master Mix (ThermoFisher, USA). The PCR targets were at the 5' end, middle, and the 3' end using specifically

Table I
Primers used in the RT-qPCR.

Target	Forward Primer	Reverse Primer
1000-A	5'-CAACCGGTGTGATCAGGACA-3'	5'-AGGACAGTCCGCATAAGCAC-3'
1000-B	5'-TACCAGCGCTTCTGTACGAC-3'	5'-GAGCTGTATCCGTGCCGTAA-3'
500-A	5'-TCGCAGGCCTAATACGTGTC-3'	5'-CGTGAATCTCGGAGCGGTAA-3'
500-B 3'end	5'-GGGTAGCGATTTAACGACTCG-3'	5'-CAGAGCCTGCCTTATCGTGA-3'
500-B middle	5'-CCGAACGCTACGTGACGATA-3'	5'-ATCTACATGTTCCGTGCGCA-3'
500-B 5'end	5'-AGACTAAATCTCGGCGTCGG-3'	5'-TAGATAGGGTCCGCATGACG-3'
500-C 3'end	5'-GCACGACCGAATTATGCACC-3'	5'-AACCACTGACGTGAGCGATT-3'
500-C middle	5'-TAGACGCGCCTTACTCCTCT-3'	5'-TAGTGGAGCTCGCGGATTTG-3'
500-C 5'end	5'-GGACTAAACGCACTGAATACCG-3'	5'-ATCGCCCGTACTATCCGGTA-3'

designed primers (Table I). To eliminate manual error in purification, standard RNA 500-A was also quantified using a primer (Table I). Calibration curve was calculated by quantifying undegraded 10^{-1} and 10^{-2} dilutions of standard RNA solution. Survival rates of each region were calculated from real-time qPCR results and corrected by quantifying standard RNA 500-A.

Results

Industrially available standard mRNA was used for quality control of prepared total RNA from *S. cerevisiae* and *E. coli*. We evaluated the yield, inhibitory effect, and degree of degradation using the standard RNA.

Before RNA extraction, standard RNA of known concentration was added into the sample tubes. We evaluated the mRNA yield, by tracing the amount of added standard RNA during the experiment. We added standard RNA during all key steps in the protocol. Total RNA was extracted from *S. cerevisiae* and *E. coli* using the hot phenol method (Sambrook et al. 1989). The hot phenol protocol proceeds in three main steps. The first step is cell disruption using phenol and SDS, the second step is purification using chloroform, and the third step is ethanol precipitation. We added different standard RNA before all three steps. Standard RNA 1000-A was added to all suspensions before reconstituting the samples, standard RNA 1000-B was added before purification using chloroform, and 500-A was added before ethanol precipitation. We measured the residual standard RNA by real-time qPCR and calculated the RNA yield.

Fig. 1 shows the yield of standard RNA in each RNA extraction step. In RNA extraction from *S. cerevisiae*, the yield of standard RNA at the cell disruption step was approximately 35%, approximately 60% after purification using chloroform, and approximately 100% after ethanol precipitation. For RNA extraction from *E. coli*, the yield of standard RNA at the cell disruption step was approximately 5%, approximately 60% after purification using chloroform, and approximately 100% after ethanol precipitation, similarly to that observed for *S. cerevisiae*.

Table II
Inhibition of RT-qPCR by RNA extract solutions from *S. cerevisiae* and *E. coli* were evaluated using standard RNAs (%).

Species	1000-A	1000-B	500-A
S. cerevisiae	−1.1 ± 4.8	50.4 ± 4.0	0.3 ± 3.1
E. coli	7.0 ± 3.2	44.6 ± 3.0	27.7 ± 2.8

To evaluate the inhibitory effect caused by contaminants, we re-added standard RNA at the same concentrations to the extracted RNA solutions and measured. Table II shows the inhibitory effect of RNA extract solution from *S. cerevisiae* and *E. coli* by RT-qPCR. Values closer to 0 denote lower inhibitory effects for RT-qPCR, whereas values closer to 100 denote higher inhibitory effect. Inhibitory effect of RNA-extract solution from *S. cerevisiae* using standard RNA 1000-A and 500-A was almost 0% and that with 1000-B was approximately 50%. For *E. coli*, inhibitory effect using standard RNA 1000-A, 1000-B, and 500-A was approximately 10%, 50%, and 30%, respectively. Standard RNA 1000-B showed higher inhibition effect for RT-qPCR than 1000-A and 500-A for both organisms.

We evaluated RNA degradation using standard RNAs. Standard RNA 500-B and 500-C were degraded

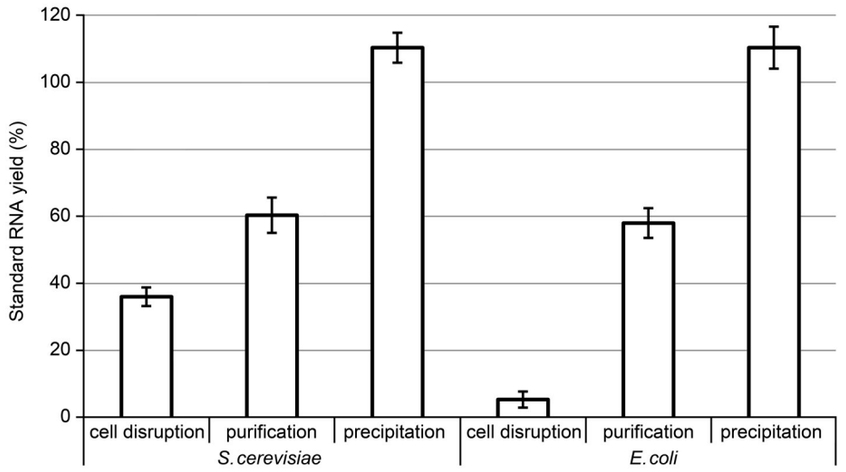

Fig. 1. RNA yields from *S. cerevisiae* and *E. coli* with hot phenol RNA extraction method using standard RNAs. Standard RNA yields were calculated based on the RT-qPCR result. Cell disruption (using phenol and SDS) was evaluated using standard RNA 1000-A. Purification (using chloroform) was evaluated using standard RNA 1000-B. Precipitation (using ethanol) was evaluated using standard RNA 500-A.

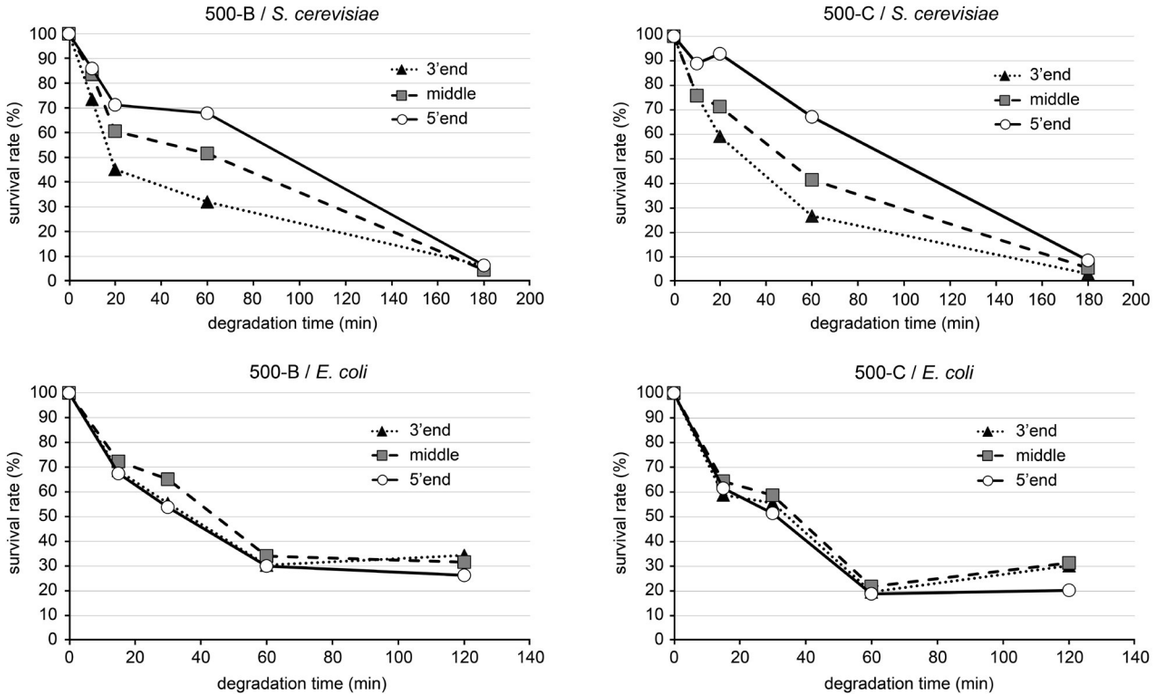

Fig. 2. Degradation of standard RNAs 500-B or 500-C by *S. cerevisiae* or *E. coli* crude extract.

by *E. coli* or *S. cerevisiae* crude extract, and the survival rate was evaluated by RT-qPCR; RNA was quantified by measuring PCR products that amplified the targeted RNA region. We quantified three regions (5' end, middle portion, and 3' end) using RT-qPCR.

Fig. 2 shows the survival rate of each region on both standard RNAs over time. We discovered that RNA degradation by *S. cerevisiae* crude extract was apparently biased toward the 3' end and was easier to degrade it than the 5' end. *E. coli* exhibited no bias. To conform the accuracy, we measured the survival rate again in three samples when the survival rates of each 3' end region were about 50%. Fig. 3 shows the quantified survival rate for triplicates of each region on both standard RNAs degraded by *S. cerevisiae* crude extract for 20 min and by *E. coli* crude extract for 30 min. In *S. cerevisiae* crude extract, the 3' end was degraded easily, and the 5' end was barely degraded in both standard RNA. For degradation by *E. coli* crude extract, no difference was observed in the degree of degradation of both ends region in both standard RNA.

We attempted to evaluate the standard RNA quality of samples degraded by *S. cerevisiae* crude extract. Table III shows degradation of standard RNA 500-B and 500-C by *S. cerevisiae* crude extract and the ratio of 3' end to 5' end survival rate. Correlation was observed between the total degradation value and the ratio of 3' end to 5' end, until RNA was degraded by approximately 50%. This value of degradation should not be used for further analysis, and, thus, the ratio of 3' end to 5' end can be used as the value for determination of the quality of RNA. Moreover, the 3' end is more prone to degradation than the 5' end, as demonstrated by quantifying RNA degradation by measurement of the 3' end of standard RNA.

We demonstrated that RNA can be easier degraded at the 3' end than the 5' end by *S. cerevisiae* crude extract, indicating that 3' to 5' exotype RNase activity

Table III
Survival of 3' end and 5' end and the ratio of 3' end to 5' end regions of standard RNAs after degradation with *S. cerevisiae* RNA crude extract.

Standard RNA	500-B					500-C				
Degradation time (min)	0	10	20	60	180	0	10	20	60	180
3' end (survival %)	100	73.5	45.2	32.1	6.3	100	76	59.2	26.7	3.2
5' end (survival %)	100	85.9	71.2	67.9	6.4	100	88.9	92.9	67.2	8.6
3' end /5' end (ratio)	1	0.86	0.63	0.47	0.99	1	0.86	0.64	0.4	0.37

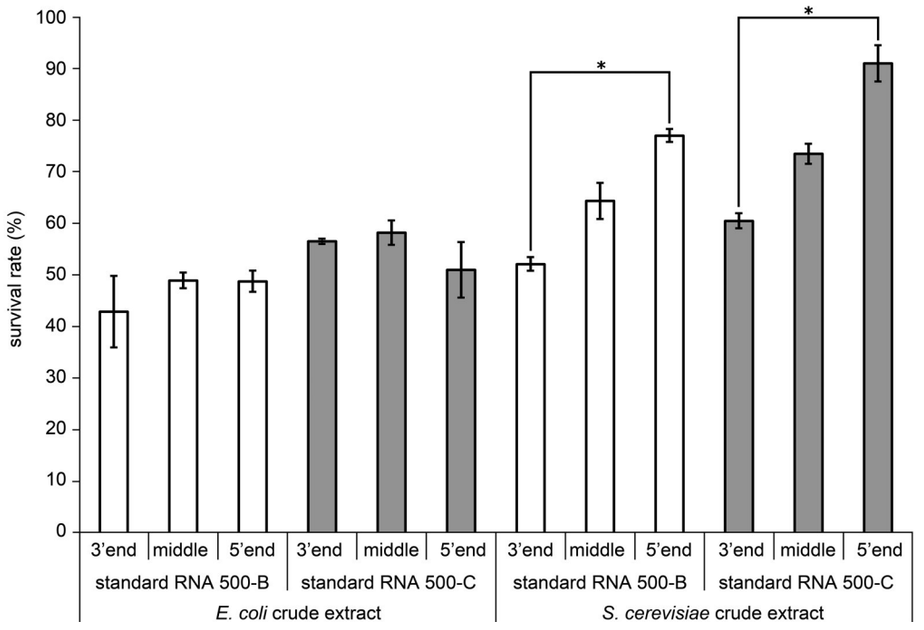

Fig. 3. The different degree of RNA degradations depends on the RNA regions in *S. cerevisiae* crude extract. Standard RNAs were degraded by *E. coli* crude extract at 30 min and by *S. cerevisiae* crude extract at 20 min. *, $p < 0.001$, n = 3, t-test.

is stronger in *S. cerevisiae*. Furthermore, single-stranded RNA can be more easily degraded than double-stranded RNA by *S. cerevisiae* RNase, as observed in this experiment from the degree of degradation of standard RNA 500-B that was higher than that of standard RNA 500-C (Fig. 2). Standard RNA 500-B tends to form a single strand, whereas standard RNA 500-C tends to form a double strand. We showed that the degree of RNA degradation by *S. cerevisiae* RNase is different for each RNA region and structure, suggesting that the prepared RNA were one-dimensional structures due to degradation.

Discussion

We calculated the yield of mRNA in each step for both *S. cerevisiae* and *E. coli*. We found that the final yield of standard RNA 1000-A from *S. cerevisiae* was approximately 35%, whereas that from *E. coli* was approximately 5%. Hot phenol was added after reconstituting the cell suspensions by thawing, which could have led to degradation of standard RNA. Lower yield of RNA from *E. coli* can be due to the higher activity of RNase, or RNase may have not affected the cells. We confirmed that ethanol precipitation is a method that benefits the final yield.

This information could fast-track the development of advanced preparation procedures. The use of standard RNA has the potential to evaluate and normalize the differences in yield of RNA from different organisms and to elucidate the reason behind low yields from specific organisms. The mRNA yield is usually not considered for determining the quality as the yield can be easily increased by increasing the volume of biological sample used to extract mRNA. Biological resources are often limited, and clinical samples and the samples obtained from the crime site are often in minute quantities, so the cost for performing forensic analysis is not economical (Georgiadis et al. 2015). Intestinal jejunum and skin are representative organs that present difficulty in mRNA extraction (Berglund et al. 2007; Heumüller-Klug et al. 2015). Thus, standard RNA may contribute toward advancing the quality of mRNA yield from these organs.

The extracted RNA may contain impurities that cause inhibitory effect on enzymatic reactions, leading to false positive results (Kashofer et al. 2013). We attempted to evaluate inhibition for real-time qPCR using standard RNA but result was incorrect. The possible reason for this result may be that the sequences of standard RNA were affected by RT-qPCR. Standard RNA is an external standard, and because the exact concentration is known, we may directly evaluate inhibitory effect. The direct evaluation of inhibitory effect disagrees with the lack of manual skill in this experiment. Furthermore, it should be noted that standard RNA may exert an inhibitory effect by interaction with secondary structures and other nucleic acids (Bustin et al. 2009).

We evaluated RNA degradation using standard RNAs. Standard RNAs were degraded by *E. coli* or

S. cerevisiae crude extract, and quantified by measuring PCR products that amplified the targeted RNA region. However, because the degree of degradation was different for each RNA region owing to cells condition and RNA structure, the quantified RNA measurement by RT-qPCR may not be a fool-proof method. RNA degradation in the cell differs among different species (Cannistraro and Kennell 1991). It has been demonstrated that bacteria, e.g., *E. coli* and *Bacillus subtilis*, degrade RNA by primarily employing endo-type RNases (Lehnik-Habrink et al. 2012; Hui et al 2014), whereas eukaryotic cells, e.g., *S. cerevisiae*, degrade RNA by employing exo-type RNases in exosomes (Szczesny et al. 2012). Therefore, if the extracted RNAs were degraded by RNase, particularly in eukaryotic cells, there is a chance that the degree of degradation was different for each RNA region.

To confirm one-dimensional degradation and to evaluate RNA degradation, standard RNAs are suitable. In conclusion, we propose the following quality control method for RNA degradation: first, standard RNA must be added during the procedures of RNA isolation; secondly, RNA degradation must be evaluated by comparing the structure and the ratio of the 5' end and 3' end of standard RNA. Furthermore, the quality of prepared RNA may be evaluated by measuring the 3' end of the standard RNA as it is more prone to degradation than the 5' end. Hence, RNA degradation quality control can be evaluated by comparing the 5' end and the 3' end of standard RNA for samples that exhibit biased RNA degradation. Further studies are required in other types of cells, including blood cells, visceral cells, and cultured cells.

Acknowledgments

This work was supported by JSPS KAKENHI Grant Number JP25670282. We would like to thank Editage (www.editage.jp) for English language editing.

Literature

Berglund SR, Schwietert CW, Jones AA, Stern RL, Lehmann J, Goldberg Z. 2007. Optimized methodology for sequential extraction of RNA and protein from small human skin biopsies. J Invest Dermatol. 127:349–353.

Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, et al. 2009. The MIQE guidelines: Minimum information for publication of quantitative real-time PCR experiments. Clin Chem. 55:611–622.

Cannistraro VJ, Kennell D. 1991. RNase I, a form of RNase I, and mRNA degradation in *Escherichia coli*. J Bacteriol. 173:4653–4659.

Feng H, Zhang X, Zhang C. 2015. mRIN for direct assessment of genome-wide and gene-specific mRNA integrity from large-scale RNA-sequencing data. Nat Commun. 6:7816.

Georgiadis AP, Kishore A, Zorrilla M, Jaffe TM, Sanfilippo JS, Volk E, Rajkovic A, Yatsenko AN. 2015. High quality RNA in semen and sperm: isolation, analysis and potential application in clinical testing. J Urol. 193:352–359.

Heumüller-Klug S, Sticht C, Kaiser K, Wink E, Hagl C, Wessel L, Schäfer KH. 2015. Degradation of intestinal mRNA: A matter of treatment. World J Gastroenterol. 21:3499–3508.

Hui MP, Foley PL, Belasco JG. 2014. Messenger RNA degradation in bacterial cells. Annu Rev Genet. 48:537–559.

Imbeaud S, Graudens E, Boulanger V, Barlet X, Zaborski P, Eveno E, Mueller O, Schroeder A, Auffray C. 2005. Towards standardization of RNA quality assessment using user-independent classifiers of microcapillary electrophoresis traces. Nucleic Acids Res. 33:e56.

Kashofer K., Viertler C, Pichler M, Zatloukal K. 2013. Quality control of RNA preservation and extraction from paraffin-embedded tissue: implications for RT-PCR and microarray analysis. PLoS One. 8:e70714.

Lehnik-Habrink M, Lewis RJ, Mäder U, Stülke J. 2012. RNA degradation in Bacillus subtilis: an interplay of essential endo- and exoribonucleases. Mol Microbiol. 84:1005–1017.

Li X, Nair A, Wang S, Wang L. 2015. Quality control of RNA-seq experiments. Methods Mol Biol. 1269:137–146.

Manchester KL. 1996. Use of UV methods for measurement of protein and nucleic acid concentrations. Biotechniques. 20:968–970.

Pionzio AM, McCord BR. 2014. The effect of internal control sequence and length on the response to PCR inhibition in real-time PCR quantitation. Forensic Sci Int Genet. 9:55–60.

Qin H, Chen X, Tang Y, Hou H, Sheng R, Shen J. 2016. Modified method for the extraction of mRNA from pabby soils. Biotechnol Lett. 38:2163–2167.

Sambrook J, Fritsch EF, Maniatis T. 1989. Molecular Cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press.

Szczesny RJ, Borowski LS, Malecki M, Wojcik MA, Stepien PP, Golik P. 2012. RNA degradation in Yeast and Human mitochondria. Biochim Biophys Acta. 1819:1027–1034.

Takahashi J, Takatsu A, Iwahashi H. 2013. Evaluation for integrity of extracted RNA by reference material of RNA. J Med Diagn Meth. 2:128.

Tong W, Lucas AB, Shippy R, Fan X, Fang H, Hong H, Orr MS, Chu TM, Guo X, et al. 2006. Evaluation of external RNA controls for the assessment of microarray performance. Nat Biotechnol. 24:1132–1139.

Wang Z, Gerstein M, Snyder M. 2009. RNA-Seq a revolutionary tool for transcriptomics. Nat Rev Genetics. 10:57–63.

Polish Journal of Microbiology
2018, Vol. 67, No 3, 355–363
https://doi.org/10.21307/pjm-2018-045

ORIGINAL PAPER

Improved Production of Recombinant Human β-NGF in *Escherichia coli* – a Bioreactor Scale Study

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Submitted 22 March 2018, revised 4 May 2018, accepted 16 June 2018

Abstract

Human nerve growth factor β (β-NGF) is considered a major therapeutic agent for treatment of neurodegenerative diseases. We have previously reported the optimized conditions for β-NGF overproduction in *Escherichia coli* in a shake-flask culture. In this study the optimal %DO (dissolved oxygen) and post induction temperature values for improved production of β-NGF were found in the bioreactor scale using response surface methodology (RSM) as the most common statistical method. Also, for further enhancement of the yield, different post-induction periods of time were selected for testing. In all experiments, the productivity level and bacterial cell growth were evaluated by western blotting technique and monitoring of absorbance at 600 nm, respectively. Our results indicated that %DO, the post-induction time and temperature have significant effects on the production of β-NGF. After 2 hours of induction, the low post induction temperature of 32°C and 20% DO were used to increase the production of β-NGF in a 5-l bioreactor. Another important result obtained in this study was that the improved β-NGF production was not achieved at highest dry cell weigh or highest cell growth. These results are definitely of importance for industrial β-NGF production.

Key words: bioreactor, RSM, β-NGF over production, *E. coli*

Introduction

NGF (Nerve Growth Factor) consists of three subunits, α, β and γ; among them β subunit is responsible for NGF biological activity. The β subunit is a dimer of two similar monomers containing cysteine knot motif, which is formed by three disulfide bonds (Wiesmann and De Vos 2001). β-NGF plays a fundamental role in development and survival of nervous system; so, it can be used as a therapeutic agent for the treatment of neurodegenerative diseases (Heese et al. 2006). NGF can be extracted from its natural source, the male mice submaxillar glands, by different methods of chromatography but it is unsuitable for clinical uses because contains heterogeneous mixtures of partially degraded dimmers (Bocchini and Angeletti 1969). Nowadays many studies have attempted to produce it as recombinant protein using different eukaryotic and prokaryotic hosts (Kurokawa et al. 2001; Choi and Lee 2004; Fan and Lou 2010).

Production of recombinant proteins in prokaryotic hosts is cost effective; among them *E. coli* is widely used expression host both in laboratory and industrial scale (Rosano and Ceccarelli 2014). Today many strategies have been introduced to increase the expression level in *E. coli*. In general, expression can be optimized by varying cultivation media, the proper selection of *E. coli* strain, induction conditions such as time and temperature of induction and the type of promoter and inducer concentration (Schumann and Ferreira 2004; Saez and Vincentelli 2014; Tegel et al. 2011). However, protein expression and production in industrial scale using a bioreactor requires special consideration. Previous studies have reported the significant effects of oxygen level, aeration and agitation rate on the growth rate in liquid medium of bioreactor (Zhong 2010; Wang et al. 2016). Agitation or shaking of culture is necessary to maintain a uniform environment without formation of bacterial aggregates throughout the bioreactor contents. Adequate agitation and aeration are also important

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factors in the success of protein expression (Thiry and Cingolani 2002; Lee et al. 2013).

The aim of this work was to optimize the fermentation parameters to obtain the highest β-NGF production in the bioreactor scale. Therefore, conditions of the fermentation process in the 5-l batch bioreactor were optimized to maximize the yield of the active protein. Response Surface Methodology (RSM) as the most common statistical method for optimization of various biochemical processes (Elibol and Ozer 2002; Wejse et al. 2003; Rui et al. 2009) was used to optimize the induction parameters and dissolved oxygen level (or different agitation rate) for β-NGF over production.

Experimental

Materials and Methods

Reagents. Unless otherwise specified, all reagents were purchased from Merck Company (Germany).

Bacterial strains and vector. The *E. coli* BL21 (DE3) carrying T7 RNA polymerase gene with genotype of *E. coli* B F^- *dcm ompT hsdS*(rB^- mB^-) *gal* λ(DE3) was used as the host strain throughout this study. The β-NGF cDNA was cloned into the expression vector pET39b (+) (Novagen, USA). The resulting vector was transformed to BL21 (DE3) strain using heat shock procedure (Sambrook and Russell 2001).

Culture media. The medium for primary and secondary seed cultures was Luria-Bertani (LB) medium (Sigma, USA) containing tryptone 10 g/l, yeast extract 5 g/l and NaCl 10 g/l. Kanamycin disulphate (Sigma, USA) at a final concentration of 50 μg/ml was added as selective factor to primary and secondary seed cultures. The medium for batch culture was SOB medium: tryptone 20 g/l, yeast extract 5 g/l, NaCl 0.5 g/l, KCl 0.186 g/l, $MgCl_2$ 0.952 g/l and $MgSO_4$ 1.204 g/l.

Culture conditions. The primary seed culture was prepared by transferring a single colony to 5 ml LB medium and incubation for 12 h at 37°C and 200 rpm. The secondary seed culture was prepared by incubating a 100 ml fresh LB medium with 1% (v/v) of the primary seed culture for 12 h at 37°C and 200 rpm.

The batch fermentation was performed in a 5-l bioreactor (Sabaferm110, Zist Farayand Sanat, Iran) containing 2 l medium inoculated with 100 ml secondary seed culture (5% v/v). The pH was kept constant throughout the experiment at 7.0 by the automatic addition of 2*N* NaOH or 2*N* HCl solution. The dissolved oxygen (DO) was maintained at different levels by changing the agitation speed (rotation speed of the mixer). The constant aeration speed of 1vvm measured and controlled by rotameter was used throughout the batch fermentation. The sterile compressed air was delivered to the bioreactor tank after passing through a cellulose filter.

Optimization of β-NGF production in the bioreactor scale. The process of β-NGF production was conducted in 5-l bioreactor. In each individual experiment, protein expression was initiated by addition of the filter sterilized solution of IPTG (isopropyl β-D-1-thiogalactopyranoside) at a final concentration of 0.3 mM when OD_{600nm} reached the value of 0.6. After desired time of production, cells were harvested by centrifugation at 5 000 g for 15 min. Subsequently, the cells were re-suspended in 8 M urea and broken by an ultrasonic disrupter. After overnight incubation at 37°C, the debris was removed by centrifugation at 12 000 g for 30 min. Finally, the supernatant was collected and used for western blotting analysis.

Determination of the best post induction time. To determine the best post induction time to achieve the highest β-NGF production, the experiments were done at constant and controlled automatically temperature of 37 ± 0.5°C and pH 7.0 ± 0.1. Also, the aeration speed and agitation speed were monitored at 1vvm and 300 rpm, respectively. After induction of the promoter by IPTG as mentioned above, the cells were grown for additional 2, 4, 6, 8, 10 and 12 h. Fermentation samples were collected to extract the proteins in 2 h intervals.

Experimental design and optimization of culture conditions. Response surface methodology (RSM) based on the central composite design (CCD) was used to optimize the culture conditions for β-NGF over production.

The percentage of dissolved oxygen (DO) and the post induction temperature were chosen as the independent variables. The ranges and levels of the two variables are listed in Table I. The cell growth at 600 nm (or the dry cells weigh) and the recombinant β-NGF expression level were chosen as response (output). The experimental data obtained from the design (Table I) were analyzed by the following second order polynomial equation:

$$Y = \beta_\circ + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j$$

Where Y is the measured response, β_o, β_i, β_{ij} and β_{ii} are the regression coefficients and X_i and X_j are the independent variables in coded values.

The software package Design Expert version 11.0.3.0 (StatSoft, USA) was used to find out the interactive effect of the two variables. The significance of the model equation and model terms were evaluated by *F*-test. The fitted polynomial equations were also expressed as three-dimension surface plots to indicate the relation between the response experimental levels

Table I
The values of independent variables (post induction temperature and dissolved oxygen) and the corresponding levels used in a central composite design (normalized in –2, –1, 0, 1 and 2).

Variable	Level				
	–2	–1	0	1	2
A: Post induction temperature (T) (°C)	20	24.25	28.5	32.75	37
B: Dissolved oxygen (DO) (%)	10	20	30	40	50

Table II
The experimental design of 10 runs with two variables (post induction temperature and dissolved oxygen) and two responses (recombinant protein expression level and cell growth measured by absorbance at 600 nm).

Run	Post induction Temperature (T) (°C)		Dissolved Oxygen (DO) (%)		Response 1 Recombinant Protein Expression Level (%)	Response 2 Cell Growth (Abs_{600nm})
1	–1	24.25	–1	20	8.0 ± 0.9	1.83 ± 0.18
2	+1	32.75	–1	20	15.3 ± 1.2	2.60 ± 0.19
3	–1	24.25	+1	40	10.1 ± 1.0	1.02 ± 0.11
4	+1	32.75	+1	40	8.4 ± 0.8	5.60 ± 0.29
5	–2	20.0	0	30	4.6 ± 0.4	1.36 ± 0.11
6	+2	37.0	0	30	11.1 ± 1.1	3.40 ± 0.22
7	0	28.5	–2	10	10.0 ± 0.9	1.87 ± 0.21
8	0	28.5	+2	50	3.8 ± 0.4	2.05 ± 0.20
9	0	28.5	0	30	14.6 ± 1.2	2.45 ± 0.19
10	0	28.5	0	30	14.2 ± 1.2	2.60 ± 0.19

of each factor used. The experimental design matrix is given in Table II. Each experiment was done in different dissolved oxygen level ranging from 10 to 50%. The temperature of culture was kept constant at 37°C before induction of the promoter, but after induction different temperatures ranging from 20 to 37°C were used according to Table II. Automatic controlling of the temperature was via the water jacket. After additional cell growth for 2 h, the culture medium was collected and centrifuged for further protein extraction.

Determination of bacterial biomass. To monitor cell growth, the absorbance of the bacterial cell culture at 600 nm was measured using a spectrophotometer (Thermo, USA). The OD_{600nm} was converted to dry cell weight (g/l) based on a standard curve (Ren et al. 2013).

Protein concentration determination. The concentration of protein in the sample was determined by Bradford method using bovine albumin as standard (Bradford 1976).

Protein identification with western blotting (WB). The proteins were subjected to electrophoresis on a 15% SDS-Polyacrylamide gel according to Laemmli method (Laemmli 1970). Then the separated proteins were electro-transferred onto the nitrocellulose membrane (Millipore, USA) in a transfer buffer (25 mM Tris, 192 mM glycine, 15% methanol) at 200 mA (Burnette 1981) for 3 h. After blocking the membrane with 5% fat-free milk in Tris-buffered saline (TBS, pH 7.4), it was treated with anti-his tag monoclonal antibody (Sigma, USA) conjugated with horseradish peroxidase at a final dilution of 1:1000. Proteins were then detected using a solution of DAB (Biobasic, Canada) and hydrogen peroxide as enzyme substrates. The area of each band on the nitrocellulose membrane which represents the β-NGF expression level was calculated by densitometry analysis (using Image J software) (Schneider et al. 2012).

Protein purification and bioactivity test. The β-NGF was successfully purified using Ni^{+2}-NTA column (ABT, Spain). Purification was done in the following steps: binding, washing and dilution with the appropriate buffers, as follows: the binding buffer (50 mM NaH_2PO_4, 300 mM NaCl, pH = 8), the washing buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM Imidazole, pH = 8) and the elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 500 mM Imidazole, pH = 7), respectively. Protein purification was finally evaluated by SDS-PAGE method. To test the biological activity of the purified β-NGF, the PC12 differentiation assay was used (Greene and Tischler 1976). 50 ng/ml of commercial and purified β-NGF was added individually to PC12 cell line for seven days and their differentiation to nerve cells was monitored.

Results

Effect of post induction time on β-NGF production. It has been widely reported that induction parameters can affect protein expression level in shake-flask culture (Azaman et al. 2010; Larentis et al. 2011; Savari et al. 2015; Gholami Tilko et al. 2016). In the present study for the first time, the effect of post-induction time on β-NGF production level was investigated in a 5-l bioreactor. Batch cultivation was performed using SOB medium with constant temperature (37°C), pH (7.0), agitation speed (300 rpm) and aeration speed (1vvm) throughout the experiment. As illustrated in Fig. 1, the maximum yield of β-NGF production (% recombinant protein expression level) was obtained at 2 h post induction time and recombinant protein expression level was reduced over extended periods of time. Therefore, post induction time of 2 h was selected and used for further experiments.

Optimization of post induction temperature and % dissolved oxygen (DO) using RSM. Post induction temperature is one of most important parameters affecting the recombinant protein expression level. Also, DO is a critical factor in the over expression of recombinant proteins, especially in the bioreactor scale. Therefore, to investigate the influence of DO and post induction temperature on β-NGF production, 10 experiments were designed using RSM method. These experiments were conducted by controlling DO (10, 20, 30, 40 and 50%) and post induction temperature (20, 24.25, 28.5, 32.75 and 37°C) (Tables I and II). Experimental error was evaluated at the central point conditions of the experimental plan (28.5°C and 30% DO). The constant cultivation temperature of 37°C was used before induction of promoter in each experiment. In all experiments, the promoter of pET39b::βNGF plasmid was induced by addition of 0.3 mM IPTG, when $OD_{600\ nm}$ reached the value of 0.6. After 2 h, the total proteins produced in recombinant bacteria were extracted. Simultaneously, cell growth (measured by absorbance at 600 nm) was monitored in each case. As the main purpose of this study was production of β-NGF, we used the western blot analysis in which an anti-his tag monoclonal antibody recognized the recombinant β-NGF with his-tag tail in the mixture of total proteins extracted from *E. coli* (Fig. 2A). To evaluate the effects of two variables (temperature and %DO) on the β-NGF expression level, the areas of each band detected on the nitrocellulose membrane were calculated by densitometry analysis (using Image J software) (Table II). Different combinations of post induction temperature (20–37°C) and dissolved oxygen (10–50%) yielded in the β-NGF production level from 3.8% ± 0.4 to 15.3% ± 1.2 as illustrated in Table II. The cell growth, which ranged from 1.02 ± 0.11 to 5.60 ± 0.29 (resulting in dry cell weight of 0.367 and 2.016 g/l) is also presented in Table II.

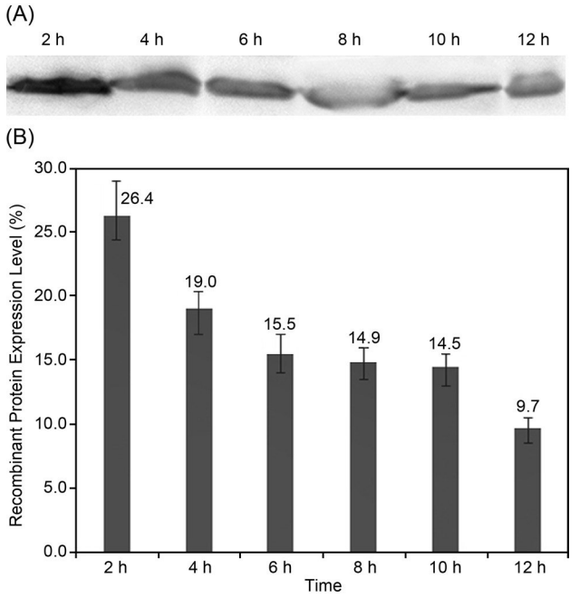

Fig. 1. The recombinant protein expression level analysis in different post induction times. (A) Western blot analysis of the proteins extracted from recombinant bacteria carrying pET39::β-NGF plasmid using anti-histag. HRP antibody. (B) % recombinant protein expression level measured by ImageJ software. All experiments were repeated three times under the identical experimental conditions.

As illustrated in Table II and Fig. 2, high β-NGF expression levels were observed in Runs 2 and 9 (15.3% ± 1.2 and 14.6% ± 1.2, respectively). Run 10 was a repetition of Run 9 and resulted in the same value. In these runs, the cell growth (or dry cell weight) was not at maximum level. In other words, the increased cell growth was not necessarily accompanied by an increase in recombinant production level. It is necessary to mention that the highest value of β-NGF production was obtained at post induction temperature of 32.75°C and % DO of 20.

In contrast, the lowest value of β-NGF production level (3.8% ± 0.4) was obtained at post induction temperature of 28.5°C and 50% DO (Run 8). When comparing the runs 8 and 9 with constant post induction temperature (28.5°C) and different %DO (also runs 2 and 4 with constant post induction temperature of 32.75°C and different %DO) it could be observed that the recombinant β-NGF production was significantly reduced at high degree of DO. It can be a result of high agitation speed because higher speed of stirring could cause a breakdown of the cells (Banerjee et al. 2009).

Analysis of variance. The recombinant β-NGF production was the most important response measured in this study. Interaction of two parameters examined in this study (post induction temperature and %DO) had pronounced effect on the β-NGF production indi-

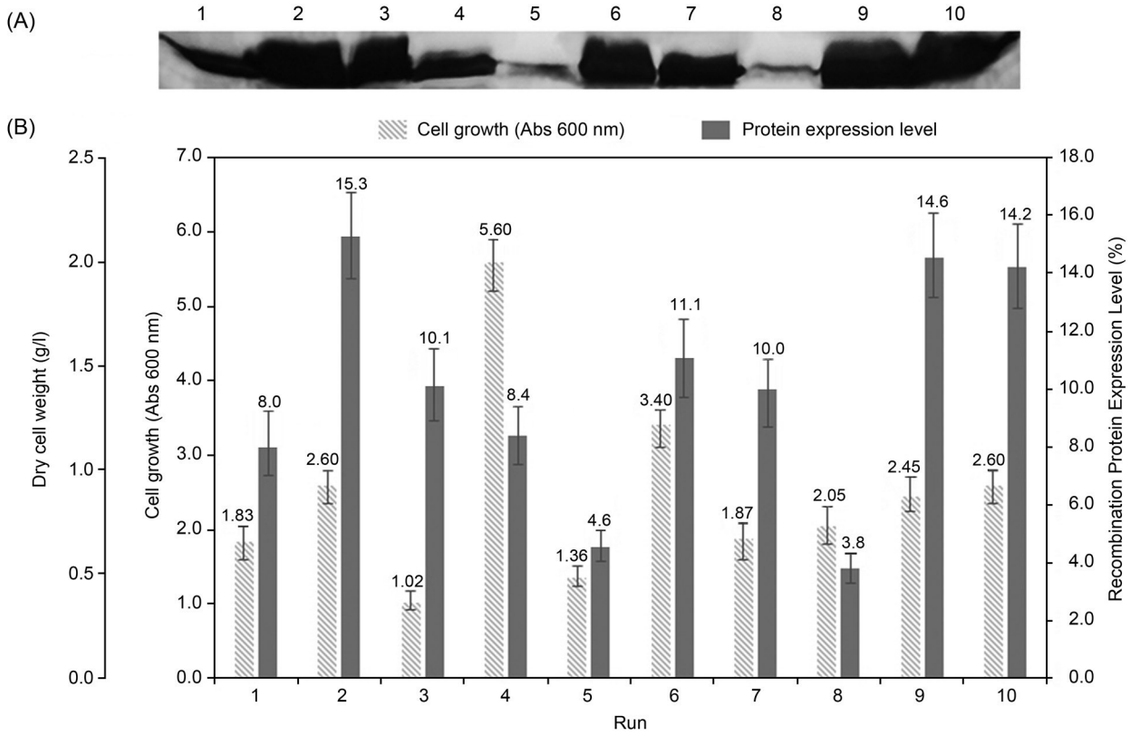

Fig. 2. The recombinant protein expression level analysis in different % DO and post induction temperature. (A) Western blot analysis of the proteins extracted from recombinant bacteria carrying pET39::β-NGF plasmid using anti-histag.HRP antibody. (B) % recombinant protein expression level measured by ImageJ software and comparison with the cell growth measured by absorbance at 600 nm. All experiments were repeated three times under the identical experimental conditions.

cating the importance of these factors for enhancement of the recombinant protein production. The response under different combinations was analyzed by ANOVA technique (Table III). The model *F*-value of 120.36 from ANOVA of the recombinant protein expression level indicated that this model was significant. The *p* values

Table III
Parameter estimates and analysis of variance (ANOVA) of the model for recombinant production of β-NGF in *E. coli* and for cell growth.

	Source of variation	Degree of freedom	Sum of squares	Mean squares	*F*-value	*p* value (Probe > *F*)
Recombinant Protein Expression Level (%)	Model	5	141.50	28.30	120.36	0.0002
	A-Temperature (°C)	1	29.14	29.14	123.94	0.0004
	B-Dissolved Oxygen (%)	1	24.65	24.65	104.85	0.0005
	AB	1	20.25	20.25	86.13	0.0007
	A^2	1	42.94	42.94	182.62	0.0002
	B^2	1	56.17	56.17	238.90	0.0001
	Error	1	0.058	0.058	–	–
	Total	9	142.44	–	–	–
Cell Growth (Abs 600 nm)	Model	3	11.58	3.86	6.82	0.0232
	A-Temperature (°C)	1	7.41	7.41	13.10	0.0111
	B-Dissolved Oxygen (%)	1	0.54	0.54	0.96	0.3655
	AB	1	3.63	3.63	6.41	0.0445
	Error	1	0.011	0.011	–	–
	Total	9	14.98	–	–	–

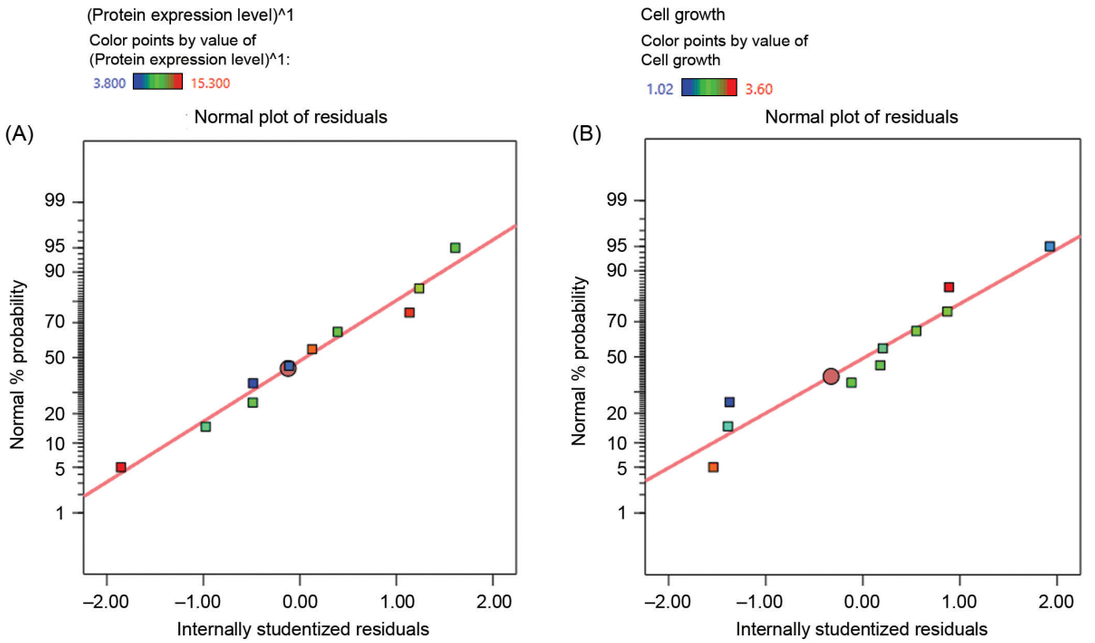

Fig. 3. Normal (%) probability plot of the studentized residuals for the model of recombinant β-NGF production (A) and cell growth (B).

were used as a tool to test the significance of each coefficient. The smaller the magnitude of *p* value, the corresponding coefficient was more significant. Also, a *p* value less than 0.05 indicated that the model terms were significant. The *p* values of terms presented in Table III indicated that both terms (A and B) had a significant influence on β-NGF production. In addition, the model is significant because *p* value is less than 0.05 (probe > F is 0.0002).

The other response was cell growth measured by absorbance at 600 nm and the effects of two parameters (post induction temperature and %DO) on this parameter were also examined. The model *F*-value of 6.82 from ANOVA of cell growth indicated that this model was also significant. The ANOVA indicated that this model and term of A (post induction temperature) were significant (*p* value < 0.05) but %DO did not significantly influence cell growth.

Model diagnostics. The normal (%) probability plot of the studentized residuals is a major tool to detect that errors are normally distributed and independent of each other (Luzier 1992). It can be seen from Fig. 3 that the errors of the models for the recombinant β-NGF production were normally distributed and insignificant.

Optimization of the process. The response surface plots and their corresponding contour plots described by the second-order polynomial equation were illustrated in Fig. 4. Each contour curve represents an infinite number of combinations of two tested variables. Fig. 4A and 4B indicate that the interaction of post induction temperature and %DO was significant. The β-NGF production increased with both post induction temperature and %DO till certain point and then decreased. The best post induction temperature and %DO for the maximum β-NGF production were 32°C and 20%, respectively. Fig. 4C and 4D show that post induction temperature had positive effect on cell growth but the effects of % DO on cell growth was not significant.

Large scale purification of β-NGF and bioactivity test. It must be noted that the β-NGF produced by pET39b::βNGF vector has his-tag tail; therefore it was purified using an affinity chromatography column (Ni^{+2}-NTA agarose). To evaluate whether the purified β-NGF is functional or not, differentiation of PC12 cell line in the presence of the purified β-NGF was studied. Our data showed that the purified β-NGF was capable of differentiate PC12 cell line to the nerve cells similarly to the standard β-NGF (data not shown) (Hajihassan et al. 2017).

Discussion

Nowadays, recombinant production of functional proteins is in high demand in modern biotechnology. *E. coli* is the most widely used expression host employed to produce many recombinant proteins. Optimization of culture conditions and induction parameters allows us to achieve the high quantity of the interest protein in this valuable host. Most of the studies in this area are in a shake-flask culture (Azaman et al. 2010; Lar-

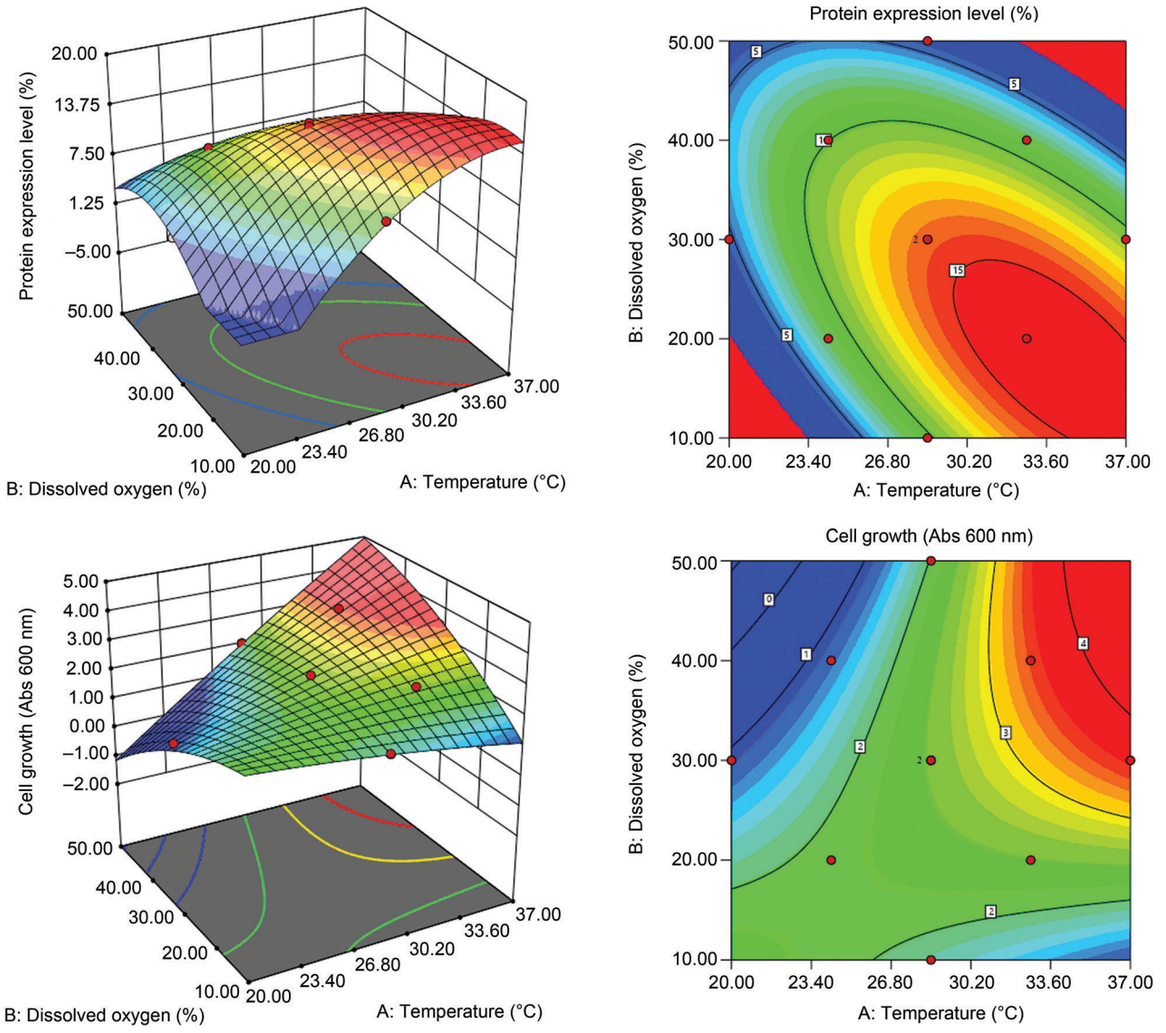

Fig. 4. Response surface plots and their counter plots show the effect of post induction temperature and % dissolved oxygen on the protein (β-NGF) expression level (A and B) and cell growth (C and D).

entis et al. 2011; Papaneophytou and Kontopidis 2012; Papaneophytou et al. 2013; Savari et al. 2015; Gholami Tilko et al. 2016). For example, in a survey recently conducted by our laboratory optimization of recombinant β-NGF production in shake-flask culture was done (Gholami Tilko et al. 2016). In the above study, the optimum post induction time and temperature were 2 h and 25°C, respectively. However, cell growth and protein production in the bioreactor scale are influenced by different parameters affecting each other (Zhong 2010). Since very few studies have been done to optimize protein production, especially recombinant proteins in industrial scale, our aim in this study was to find out the optimal conditions for high level production of recombinant β-NGF in *E. coli* grown in a 5-l bioreactor. Among the most important factors influencing the recombinant protein production level, the post induction time, post induction temperature and %DO play critical roles. Optimization of these parameters results in higher productivity and reduces cost of fermentation. It is of interest to note that the best induction time can be established for each recombinant protein by a trial and error. However, in many reports the expression is reduced over extended induction times, and this phenomenon is likely due to the recombinant protein degradation by bacterial proteinases (Joshi and Puri 2005; Choi et al. 2006; Papaneophytou et al. 2013; Gholami Tilko et al. 2016). In this study to produce high level of recombinant β-NGF in the bioreactor scale, the post induction times between 2 to 12 hours was examined. According to our results, the post induction time of 2 h is optimal for improved recombinant β-NGF production level.

Another factor that influences the expression level is post induction temperature. As the best cultivation temperature for *E. coli* is between 35–37°C, lowering the

temperature causes a lower growth rate. On the other hand, in the case of expression of complex heterologous proteins, usually a decrease in the temperature during protein synthesis especially after IPTG induction and, consequently, slower growth rate of bacteria favor the production of the active protein form. It is also important to note that in the literature different post induction temperatures have been reported for overproduction of different heterologous proteins in the bioreactor scale or shake-flask cultures (Azaman et al. 2010; Papaneophytou and Kontopidis 2012; Morowvat et al. 2015; Savari et al. 2015; Su et al. 2015; Gholami Tilko et al. 2016). Therefore, the evaluation of the best post induction temperature for β-NGF production on bioreactor scale was one of the goals of this study. Also %DO is a critical factor in the over expression of recombinant proteins, especially in the bioreactor scale, because it can affect directly cell growth (Ram et al. 2015; Wang et al. 2016). Previous studies showed that bacterial growth and protein production were dependent on the degree of oxygenation; distribution of oxygen to the bacteria in the bioreactor was related to the intensity of aeration and stirring or agitation (Çalik et al. 2004; Kaya-Çeliker et al. 2009). On the other hand, at high degree of DO as a result of high agitation speed break down of the cells takes place (Banerjee et al. 2009). Recently, Zaslona et al. (2015) showed that production of recombinant 1,3-β-glucanase in *E. coli* increased when moderate stirring and oxygenation was applied. Therefore, to evaluate the effects of %DO and post induction temperature on β-NGF expression level and cell growth rate, we used response surface methodology (RSM) as a high effective method (Elibol and Ozer 2002; Wejse et al. 2003; Rui et al. 2009). Thus, various combinations of %DO (10–50) and post induction temperature (20–37°C) as two independent variables were examined (Tables I and II). Our results indicated that the highest β-NGF production in the bioreactor scale was obtained at post induction temperature and %DO of 32.75°C and 20%, respectively (Fig. 1). On the other hand, in this run the maximum cell growth or dry cell weigh were not obtained. It can be concluded that increasing cell growth does not necessarily increase recombinant protein expression level. The most important result obtained here was that the highest β-NGF production was achieved at moderate %DO (20%) and post induction temperature of 32°C. These results are in agreement with the previous data presented by others on the production of various proteins in *E. coli* in the bioreactor scale (Zaslona et al. 2015).

In summary, the response surface methodology was successfully used for the optimization of recombinant β-NGF production by varying the induction parameters and %DO. Our results could be beneficial for industrial production of β-NGF.

Acknowledgements

This work was supported by grants from the Scientific and Technological Department of Presidential Office (Iranian Council of Stem Cell Technology). The authors would like to acknowledge the financial support of University of Tehran for this research.

Conflict of interest

The authors declare that they have no conflict of interest.

Literature

Azaman SNA, Ramakrishnan NR, Tan JS, Rahim RA, Abdullah MP, Ariff AB. 2010. Optimization of an induction strategy for improving interferon-α2b production in the periplasm of *Escherichia coli* using response surface methodology. Biotechnol Appl Biochem. 56(4):141–150.

Banerjee A, Dubey S, Kaul P, Barse B, Piotrowski M, Banerjee UC. 2009. Enantioselective nitrilase from Pseudomonas putida: cloning, heterologous expression, and bioreactor studies. Mol Biotechnol. 41(1):35–41.

Bocchini V, Angeletti PU. 1969. The nerve growth factor: purification as a 30 000-molecular-weight protein. Proc Natl Acad Sci USA. 64(2):787–794.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 72(1–2):248–254.

Burnette WN. 1981. "Western Blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Anal Biochem. 112(2):195–203.

Çalik P, Yilgör P, Ayhan P, Demir AS. 2004. Oxygen transfer effects on recombinant benzaldehyde lyase production. Chem Eng Sci. 59(22–23):5075–5083.

Choi JH, Keum KC, Lee SY. 2006. Production of recombinant proteins by high cell density culture of *Escherichia coli*. Chem Eng Sci. 61(3):876–885.

Choi JH, Lee SY. 2004. Secretory and extracellular production of recombinant proteins using *Escherichia coli*. Appl Microbiol Biotechnol. 64(5):625–635.

Elibol M, Ozer D. 2002. Response surface analysis of lipase production by freely suspended *Rhizopus arrhizus*. Process Biochem. 38(3):367–372.

Fan BS, Lou JY. 2010. Recombinant expression of human nerve growth factor beta in rabbit bone marrow mesenchymal stem cells. Mol Biol Rep. 37(8):4083–4090.

Gholami Tilko P, Hajihassan Z, Moghimi H. 2017. Optimization of recombinant β-NGF expression in *Escherichia coli* using response surface methodology. Prep Biochem Biotechnol. 47(4):406–413.

Greene LA, Tischler AS. 1976. Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. Proc Natl Acad Sci USA. 73(7):2424–2428.

Hajihassan Z, Abdi M, Roshani Yasaghi E, Rabbani-Chadegani A. 2017. Optimization of recombinant beta-NGF purification using immobilized metal affinity chromatography. Minerva Biotecnol. 29:126–132.

Heese K, Low JW, Inoue N. 2006. Nerve growth factor, neural stem cells and Alzheimer's disease. Neurosignals. 15(1):1–12.

Joshi BH, Puri RK. 2005. Optimization of expression and purification of two biologically active chimeric fusion proteins that consist of human interleukin-13 and Pseudomonas exotoxin in *Escherichia coli*. Protein Expr Purif. 39(2):189–198.

Kaya-Çeliker H, Angardi V, Çalık P. 2009. Regulatory effects of oxygen transfer on overexpression of recombinant benzaldehyde

lyase production by *Escherichia coli* BL21 (DE3). Biotechnol J. 4(7):1066–1076.

Kurokawa Y, Yanagi H, Yura T. 2001. Overproduction of bacterial protein disulfide isomerase (DsbC) and its modulator (DsbD) markedly enhances periplasmic production of human nerve growth factor in *Escherichia coli*. J Biol Chem. 276(17):14393–14399.

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 227(5259):680–685.

Larentis AL, Argondizzo APC, Esteves GS, Jessouron E, Galler R, Medeiros MA. 2011. Cloning and optimization of induction conditions for mature PsaA (pneumococcal surface adhesin A) expression in *Escherichia coli* and recombinant protein stability during long-term storage. Protein Expr Purif. 78(1):38–47.

Lee EJ, Lee BH, Kim BK, Lee JW. 2013. Enhanced production of carboxymethylcellulase of a marine microorganism, *Bacillus subtilis* subsp. subtilis A-53 in a pilot-scaled bioreactor by a recombinant *Escherichia coli* JM109/A-53 from rice bran. Mol Biol Rep. 40(5):3609–3621.

Luzier WD. 1992. Materials derived from biomass/biodegradable materials. Proc Natl Acad Sci USA. 89(3):839–842.

Morowvat MH, Babaeipour V, Rajabi Memari H, Vahidi H. 2015. Optimization of fermentation conditions for Recombinant Human Interferon Beta production by *Escherichia coli* using the response surface methodology. Jundishapur J Microbiol. 8(4):e16236.

Papaneophytou CP, Kontopidis GA. 2012. Optimization of TNF-α overexpression in *Escherichia coli* using response surface methodology: purification of the protein and oligomerization studies. Protein Expr Purif. 86(1):35–44.

Papaneophytou CP, Rinotas V, Douni E, Kontopidis G. 2013. A statistical approach for optimization of RANKL overexpression in *Escherichia coli*: purification and characterization of the protein. Protein Expr Purif. 90(1):9–19.

Ram KS, Babu KS, Tabitha G, Rajeshwari K, Lakshmi GJ, Gowd BB, Peravali JB, Rao MS, Rao PV. 2015. High cell density cultivation for the production of industrially important engineered Bi-functional recombinant staphylokinase variant from salt inducible *Escherichia coli* GJ1158. I.J.B.S.B.T. 7:327–338.

Ren Q, Henes B, Fairhead M, Thöny-Meyer L. 2013. High level production of tyrosinase in recombinant *Escherichia coli*. BMC Biotechnol. 13(1):18–18.

Rosano GL, Ceccarelli EA. 2014. Recombinant protein expression in *Escherichia coli*: advances and challenges. Front Microbiol. 5:172.

Rui L, Wei-chang C, Wei-peng W, Wen-yan T, Xue-guang Z. 2009. Optimization of extraction technology of *Astragalus polysaccharides* by response surface methodology and its effect on CD40. Carbohydr Polym. 78(4):784–788.

Saez NJ, Vincentelli R. 2014. High-throughput expression screening and purification of recombinant proteins in *E. coli*. Methods Mol Biol. 1091:33–53.

Sambrook J, Russell DW. 2001. Molecular cloning: a laboratory manual 3rd edition. Cold Spring Harbor, New York (USA): Cold Spring-Harbour Laboratory Press.

Savari M, Zarkesh Esfahani SH, Edalati M, Biria D. 2015. Optimizing conditions for production of high levels of soluble recombinant human growth hormone using Taguchi method. Protein Expr Purif. 114:128–135.

Schneider CA, Rasband WS, Eliceiri KW. 2012. NIH Image to ImageJ: 25 years of image analysis. Nat Methods. 9(7):671–675.

Schumann W, Ferreira LCS. 2004. Production of recombinant proteins in *Escherichia coli*. Genet Mol Biol. 27(3):442–453.

Su L, Huang Y, Wu J. 2015. Enhanced production of recombinant *Escherichia coli* glutamate decarboxylase through optimization of induction strategy and addition of pyridoxine. Bioresour Technol. 198:63–69.

Tegel H, Ottosson J, Hober S. 2011. Enhancing the protein production levels in *Escherichia coli* with a strong promoter. FEBS J. 278(5):729–739.

Thiry M, Cingolani D. 2002. Optimizing scale-up fermentation processes. Trends Biotechnol. 20(3):103–105.

Wang D, Wang C, Wu H, Li Z, Ye Q. 2016. Glutathione production by recombinant Escherichia coli expressing bifunctional glutathione synthetase. J Ind Microbiol Biotechnol. 43(1):45–53.

Wejse PL, Ingvorsen K, Mortensen KK. 2003. Xylanase production by a novel halophilic bacterium increased 20-fold by response surface methodology. Enzyme Microb Technol. 32(6): 721–727.

Wiesmann C, de Vos AM. 2001. Nerve growth factor: structure and function. Cell Mol Life Sci. 58(5):748–759.

Zaslona H, Trusek-Holownia A, Radosinski L, Hennig J. 2015. Optimization and kinetic characterization of recombinant 1,3-β-glucanase production in *Escherichia coli* K-12 strain BL21/pETSD10 – a bioreactor scale study. Lett Appl Microbiol. 61(1): 36–43.

Zhong JJ. 2010. Recent advances in bioreactor engineering. Korean J Chem Eng. 27(4):1035–1041.

Polish Journal of Microbiology
2018, Vol. 67, No 3, 365–375
https://doi.org/10.21307/pjm-2018-046

ORIGINAL PAPER

The Emergence of Different Functionally Equivalent PAH Degrading Microbial Communities from a Single Soil in Liquid PAH Enrichment Cultures and Soil Microcosms Receiving PAHs with and without Bioaugmentation

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Submitted 27 November 2018, revised 16 February 2018, accepted 22 June 2018

Abstract

Polycyclic aromatic hydrocarbon (PAHs) are common soil contaminants of concern due to their toxicity toward plants, animals and microorganisms. The use of indigenous or added microbes (bioaugmentation) is commonly used for bioremediation of PAHs. In this work, the biodegradation rates and changes in the bacterial community structure were evaluated. The enrichment culture was useful for unambiguously identifying members of the soil bacterial community associated with PAH degradation and yielded a low diversity community. No significant difference in the rate of PAH degradation was observed between the microcosm receiving only PAHs or PAHs and bioaugmentation. Moreover, identical matches to the bioaugmentation inoculum were only observed at the initial stages of PAH degradation on day 8. After 22 days of incubation, the substantial degradation of all PAHs had occurred in both microcosms and the PAH contaminated soil had statistically significant increases in Alphaproteobacteria. There were also increases in Betaproteobacteria. In contrast, the PAH contaminated and bioaugmented soil was not enriched in PAH degrading Proteobacteria genera and, instead, an increase from 1.6% to 8% of the population occurred in the phylum Bacteroidetes class Flavobacteria, with *Flavobacterium* being the only identified genus. In addition, the newly discovered genus *Ohtaekwangia* increased from 0% to 3.2% of the total clones. These results indicate that the same soil microbial community can give rise to different PAH degrading consortia that are equally effective in PAH degradation efficiency. Moreover, these results suggest that the lack of efficacy of bioaugmentation in soils can be attributed to a lack of persistence of the introduced microbes, yet nonetheless may alter the microbial community that arises in response to PAH contamination in unexpected ways.

Key words: polycyclic aromatic hydrocarbon, PAH, soil microbial community, biodegradation, bioaugmentation

Introduction

PAHs contamination of soil is wide spread as a result of the use of petroleum fuels, lubricants and petrochemicals and the associated spills, aerosols, disposal. In addition, there is significant release of PAH's from natural oil reservoirs, both terrestrially and marine. A primary concern regarding PAHs is their toxicity toward plants, animals and microorganisms, with some being known or suspected carcinogens (Eisler 1987; Petry et al. 1996). Currently there are 32 PAH compounds listed as priority pollutants by the US EPA; these include naphthalene, phenanthrene, anthracene and pyrene.

PAHs of environmental concern are biodegradable, however, their rate of degradation tends to decrease with increases in ring number, ring arrangement and substitutions to the aromatic rings making many of the higher molecular weight PAHs persist in soils for extended periods (Juhasz and Naidu 2000). PAH degradation has been identified in wide a range bacteria of both Gram-positive phyla including Actinobacteria, Deinococcus-Thermus and Firmicutes, and Gram-negative phyla, including Bacteroidetes, Cyanobacteria, and all classes of Proteobacteria accept the Deltaproteobacteria (Prince et al. 2010). Nonetheless, PAH degradation in soils and sediments is complex and still poorly understood with respect to the dynamics of the

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response of the PAH degrading microbial community after PAH contamination. This can be attributed in part to the fact that these environments typically contain a variety of different PAH degrading microorganisms with different environmental niches and metabolic strategies, metabolic pathways and substrate ranges (Janssen 2006). In addition, there are other external factors as the presence of the fungi and surfactants, which can affect the microbial community (Wyrwas et al. 2013; Szczepaniak et al. 2016).

Due to their degradability PAHs are frequently removed from soils by natural attenuation or bioremediation by the addition of limiting nutrients, usually N, P and oxygen, and/or bioaugmentation by the addition of organisms capable of degrading these compounds (Samanta et al. 2002; Van Hamme et al. 2003). Bioremediation by addition of limiting nutrients has been shown to be quite effective under nutrient limited conditions (Lindstrom et al. 1991; Philp and Atlas 2005); however, the effectiveness of bioaugmentation is less predictable with no detectable improvement in PAH removal observed in many cases (Silva et al. 2009; Sayara et al. 2011; Tyagi et al. 2011). Moreover, in some cases, PAH removal is not benefited by the either addition of nutrients or exogenous microorganisms due to the fact that in such cases these are not limiting parameters (Bento et al. 2005).

The aim of this study was to evaluate changes in the microbial community in a pristine agricultural soil contaminated with the PAHs naphthalene, phenanthrene, anthracene and pyrene (hence forth referred to as PAH amended) and with the addition of PAH's in combination with bioaugmentation by the addition of an exogenous PAH degrading consortia (hence forth referred to as bioaugmented).

Experimental

Materials and Methods

PAH degrading isolates used for bioaugmentation. Five PAH degrading isolates identified as *Bacillus* sp., *Bacillus brevis*, *Bacillus sphaericus*, *Bacillus subtilis*, and *Chromobacterium* sp., isolated from hydrocarbon contaminated soils near the REPLAN oil refinery in Southeastern Brazil and from the effluent of an oil tank steam cleaning facility, were used for bioaugmentation. To verify that the strains could degrade the PAHs used in this study, cultures were inoculated separately into 30 ml of minimal salts medium (Rambeloarisoa et al. 1984), containing 30 µl of vitamin solution (Wolin et al. 1963), 9 mg of naphthalene and phenanthrene, and 6 mg of anthracene and pyrene (1 g/l total PAHs) as the carbon source. Cultures were incubated at 30°C on a rotary shaker at 150 rpm. Efficient growth was observed for all isolates used for bioaugmentation.

For inoculum preparation the selected strains were grown separately in nutrient broth medium on a rotary shaker at 150 rpm and 30°C until the desired cell density (OD_{590} nm = 1). Cell density in relation to OD was determined as colony forming units (CFU) from serial dilutions on nutrient agar plates. Each culture was centrifuged and resuspended in 0.75% saline solution and the isolates were mixed together for inoculation of the microcosms.

PAH enrichment cultures. Enrichments for PAH degraders from the soil used in the microcosms was performed in the same liquid culture as describe above for verification of the PAH degrading ability of the bioaugmentation consortia. Three grams of soil were suspended in 30 ml of the same media in sterile screw capped plastic tubes without PAHs and vortexed for 5 minutes. After vortexing the sediment allowed to settle for 10 minutes. A 10 ml aliquot of the supernatant was used to inoculate 250 ml of minimal media containing 250 mg/l of naphthalene, phenanthrene, anthracene and pyrene. Enrichments were performed in triplicate. One ml samples were taken for GC analysis as described below. After 22 days of incubation, at which time the efficient growth and degradation of all PAHs were observed in all enrichment replicates, a combined sample was processed using the MoBio soil DNA extraction kit in the same fashion as for soil DNA extraction and used for 16S rRNA gene sequence analysis as described below.

Microcosm setup. The soil used for the microcosms had no history of PAH contamination; it was collected in an area located at the Experimental Faculty of Agricultural Engineering, State University of Campinas, latitude 22°48'57" south, longitude 47°03'33" west at an average altitude of 640 m. The soil is characterized as dystrophic clayey Oxisol (Typic Haplorthox), which is a soil common to various regions of Brazil.

Microcosms were performed in duplicate. After correction of soil moisture to 55% of holding capacity and homogenization, 400 g of soil was added to 1.5 l glass jars with airtight lids. The PAHs were dissolved in toluene and 6 ml of the PAH solution was added to the surface of the PAH amended and PAH amended and bioaugmented (hence forth referred to as bioaugmented) microcosm to give the following mass of each PAH, naphthalene and phenanthrene 150 mg, anthracene, and pyrene 100 mg and 50 mg hexachlorobenzene as a non-biodegradable marker for use in calculating the percent degradation of the PAHs. The microcosms were left open for 48 h in a fume hood with high airflow to allow the toluene to evaporate. After evaporation of the toluene the soils were thoroughly mixed and inoculum was added to the bioaugmented microcosms

to give a final concentration of each PAH degrader of 10^7 CFU/g soil, after which the soils were thoroughly mixed again. A control without PAHs or inoculum was also prepared to evaluate the changes in microbial community structure over the incubation period and to evaluate the community response to the addition of PAHs and bioaugmentation.

Microcosms were incubated at room temperature and sampled every three days. At each sampling time the soils were first thoroughly mixed with a sterile spatula to aerate and homogenize the soil. One-gram samples were then taken for GC analysis and four one-gram samples were taken from different locations in the microcosm and combined for DNA extraction for construction of the 16S rRNA gene libraries.

GC analysis of PAH degradation. Samples for PAH degradation were analyzed individually for the duplicate microcosms and the average values were used for determining percent PAH degradation. The aromatic compounds were extracted from 1 g samples of soil from each microcosm at the indicated times. The samples were placed into vials containing 1 ml of dichloromethane. The vials with soil were then sonicated 10 times for 30 seconds with 30-second intervals on ice between each sonication. One gram of anhydrous Na_2SO_4 was then added and mixed into the soil to remove moisture from the sample. The dichloromethane supernatants were transferred to 1.5 ml Eppendorf tubes and centrifuged at 5 000 × g for 5 minutes at 4°C. An aliquot of the supernatant without any particulate material was transferred from the Eppendorf tubes and placed into 2 ml glass vials with screw cap lids with Teflon septa and stored at –20°C for subsequent GC analysis.

The degradation of PAHs was determined using a 1 µl aliquot of the dichloromethane extract injected in duplicate in splitless mode into a gas chromatograph (Shimadzu GC 14A), equipped with a flame ionization detector (GC-FID), and separated using an ID-BPX-5 column with fused silica as the stationary phase (25 m length × 0.22 mm ID × 0.25 µm film thickness, SGE-Australia) under a helium flow rate of 0.7 ml per minute. The injector temperature was set at 240°C and the detector temperature was set at 300°C. The oven temperature ramp rate was programmed as follows, 70°C hold for 1 minute, followed by an initial temperature ramp rate of 30°C per minute to 160°C, followed by an increase of 15°C per minute until reaching 310°C, at which time all PAHs had been eluted from the column. Percentage degradation was determined from the ratio of the peak height signal for each PAH to that of hexachlorobenzene obtained at T = 0 minus the ratio on the day of sampling divided by that obtained at T = 0, multiplied by 100. Percent standard deviation between duplicate injections was 10% or less for all samples.

Cloning, sequencing and analysis of 16S rRNA genes. Samples for 16S rRNA gene analysis were combined from replicate microcosms prior to DNA extraction and analysis. Total genomic DNA was extracted from 1 g of the combined soil samples using the Soil DNA Extraction Kit (MoBio Laboratories, USA) according to the procedure described by the manufacturer. PCR amplification of bacterial 16S rRNA genes was performed in 50 µl volumes containing 5 µl of 10X buffer with $MgCl_2$ (Eppendorf), 8 mM dNTP's (deoxyribonucleotide triphosphates), 2.5 µl of each primer, 0.5 µl of Taq polymerase (Eppendorf) and 2 µl of DNA, using the total genomic DNA. The bacteria domain specific primers 27F - 5'AGAGTTTGATCMTGGCTCAG3' and 1492R - 5'GGTTACCTTGTTACGACTT3' were used and the PCR amplification was performed using the following thermal cycling conditions: initial denaturation at 95°C for 5 min, 30 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min, extension at 72°C for 2 min, followed by a final extension at 72°C for 10 min. The amplified products were checked by electrophoresis in 2% agarose gel. All PCR amplification reactions were performed in a BioRad model iCycler. For automated sequencing, PCR products were purified using mini-columns (GFX PCR DNA and Gel Band Purification Kit, GE Health Care) and submitted to sequencing in an automated sequencer (MegaBace, GE Health Care). The sequencing reactions were performed using the DYEnamic ET Dye Terminator Cycle Sequencing Kit and MegaBace DNA Analysis Systems (GE Health Care) using the primer 338F - 5'ACTCCTACGGGAGGCAGCAG3' (Lane 1991), which targets the hyper-variable V3 region of the 6S rRNA gene.

Phylogenetic analysis and comparison of cloned libraries. The phylogenetic diversity of the soil microcosms was determined using the CLASSIFIER algorithm provided by the Ribosome Database Project (RDP) release 10 website (Wang et al. 2007; Cole et al. 2009). This algorithm uses a naive Bayesian method of comparative statistical analyses to classify bacterial 16S rRNA sequences. Identification of specific clone sequence matches to the RDP version 11 (rdp.cme.msu.edu) and the NCBI (www.ncbi.nlm.nih.gov) nucleotide sequence (nr/nt) database was performed using the RDP online sequence comparison tool SeqMatch (Cole et al. 2005), using NCBI taxonomy, and the NCBI BLASTN DNA sequence comparison program, respectively. Microbial community comparisons between the different microcosms were performed using the RDP LIBCOMPARE program, which uses the Classifier taxonomic identifications to estimate the probability of obtaining the observed difference between two data sets for a given taxon (Cole et al. 2009). The default confidence threshold of 80% was used for assigning each

taxon group. Those taxonomic groups that were identified as being significantly different at $p < 0.01$ were used to identify major community differences.

Results and Discussion

Bacterial population in the liquid PAH enrichment culture. The soil used for the microcosms was used as the inoculum for liquid PAH enrichment cultures containing 250 mg/l of naphthalene, phenanthrene, anthracene and pyrene as the sole carbon sources. The purpose of the liquid enrichment was to identify members of the soil community that emerged in response to growth in the presence PAHs as the sole carbon source. However, liquid culture is clearly a very different environment than soil and those organisms identified would also represent likely PAH degraders that had a competitive advantage in liquid culture. After 22 days all of the PAHs were substantially degraded (data not shown) and total genomic DNA was isolated for analysis of the community structure by comparative 16S rRNA gene sequence analysis. A total of 245 high quality partial bacterial 16S RNA gene sequences were obtained and analyzed using the RDP database suite of programs as described in Methods. The bacterial population of the PAH enrichment was of low diversity in comparison to those of the soil microcosms and was almost entirely composed of bacteria from the phylum Proteobacteria, which represented 91.3% of the total clones.

The Proteobacteria consisted of 19% Alphaproteobacteria, 28% Betaproteobacteria, and 53% Gamaproteobacteria. The Alphaproteobacteria were composed of *Brevundimonas* (5%), *Azospirillum* (17%), *Shingomonas* and unclassified Sphingomonadaceae (29%), and 48% were identified as members of the order Rhizobiales consisting of *Hyphomicrobium* and unclassified Hyphomicrobiaceae (14%), *Rhizobium* and unclassified Rhizobiales (20%), *Bosea* (5%) and *Phyllobacterium* (5%). The Betaproteobacteria consisted of *Bordetella* (87%), *Achromobacter* (8%) and unclassified Alcaligenaceae. The Gamaproteobacteria consisted of *Pseudoxanthomonas* (50%), *Steroidobacter* (1%), *Pseudomonas* (26%) and unclassified Pseudomonadaceae (18%, although all of these were identified as either *Pseudomonas* or *Azomonas* at confidence levels below 80%), and the remaining 5% were unclassified Gammaproteobacteria. All of these genera have been associated with PAH degradation (Wilson and Jones 1993; Vinas et al. 2005; Haritash and Kaushik 2009; Prince et al. 2010; Brown et al. 2012).

Members of the phylum Bacteroidetes were the only other dominant group in the enrichment representing 7.4% of the clones. The remaining 0.8% where unclassified bacteria. The Bacteroidetes clones consisted of 39% *Sediminibacterium* in the family Chitinophagaceae and 61% unclassified Chitinophagaceae, of these 55% were classified as *Sediminibacterium* at confidence levels ranging form 60% to 76%. *Sediminibacterium* where also found represent up to 12% of the total population in semi-continuous slurry-phase bioreactors treating PAHs (Singleton et al. 2011). In marked contrast to the soil microcosms (see below) no clones were obtained from the phylum Firmicutes, which contains the genus *Bacillus*.

Rates of PAH degradation in the PAH amended versus the PAH amended and bioaugmented soil. For the abiotic control there was effectively no depletion of any of the PAH over the course of the experiment with the exception of naphthalene, which saw a loss of approximately 50% on day 22 attributed to sublimation (data not shown). The percent degradation of the PAHs over time is shown for the PAH amended soil and the PAH amended and bioaugmented soil in Fig. 1 and 2, respectively. Both the PAH amended at the bioaugmented microcosms demonstrated effectively equal rates of degradation. Samples for microbial community analysis were taken on: i) day 1 for the

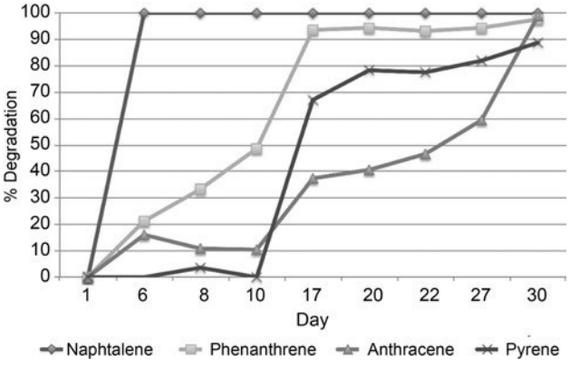

Fig. 1. Percent PAH degradation in the PAH amended soil without bioaugmentation.

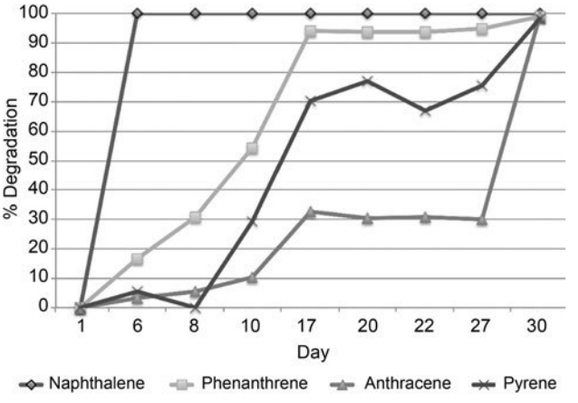

Fig. 2. Percent PAH degradation in the PAH amended and bioaugmented soil.

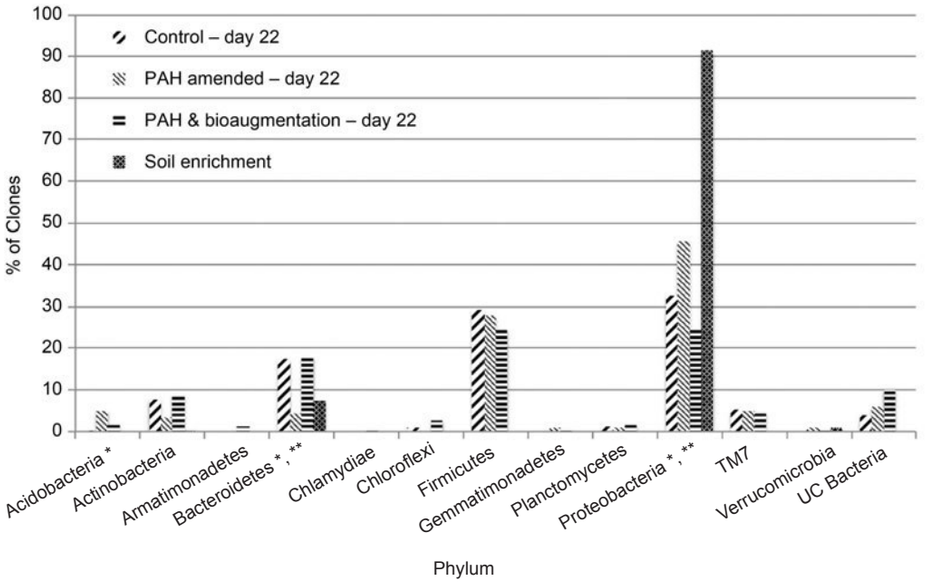

Fig. 3. Comparison of the percentage of clones at the phylum level between the unamended control, PAH amended, bioaugmented microcosms and the soil enrichment on day 22.

* Indicates those taxonomic groups that are significantly different at $p < 0.01$ between the control and the PAH amended microcosms and ** indicates those taxonomic groups that are significantly different between the PAH amended and PAH amended and bioaugmented microcosms.

unamended control; ii) day 8 from the PAH amended and the bioaugmented soils, at which point degradation of all of the PAHs had begun in both sets of microcosms; iii) day 22 for all microcosms at which point 30% or more of all of the PAHs had been degraded, with exception of naphthalene which was completely removed. By day 30 virtually all of the PAHs had been removed in both microcosms. The lack of a significant difference in degradation rates between the uninoculated and inoculated microcosms demonstrates that bioaugmentation did not improve the bioremediation of these compounds.

Characterization of the changes in microbial populations in the soil microcosms. The microbial communities were analyzed on day 1 for the unamended control, on day 8 for the PAH treated microcosms and on day 22 for all 3 sets of microcosms to assess the effect of PAH contamination and bioaugmentation. The following number of high quality sequences over 250 bp were obtained from each microcosm (number of sequences); unamended control day 1 (145), PAH amended and bioaugmented day 8 (316 and 338, respectively), and day 22 unamended control (234), PAH amended (116) and the PAH amended and bioaugmented soil (188).

The cut off for taxons that were scored as statistically significant with respect to their relative abundance between microcosms was set at $p < 0.01$ and was used to identify meaningful differences between populations by the Libcompare program. There were a number of sequences that could not be classified beyond a certain taxonomic level and are designated UC (unclassified beyond that taxonomic level) in the following figures.

Comparison of the microbial communities of the unamended control and the PAH amended microcosms. The unamended control was sampled at the initiation of the microcosms and on day 22, and no statistically significant differences were observed (data not shown) indicating that exposure to microcosms conditions did not induce significant changes in the soil microbial population. The relative abundance of the identified phyla in all microcosms as well as the PAH enrichment culture on day 22 is shown in Fig. 3.

Comparing the bacteria from the PAH amended to the unamended control soil shows statistically significant differences. This fact was expected due to contamination of the soil by PHAs causes significant changes in the soil bacterial community (Szczepaniak et al. 2016). Microorganisms, mainly bacteria, are the prevailing organisms in soils. Bacteria as heterotrophs could mineralize contaminants, e.g. hydrocarbon (Megharaj et al. 2011; Abbasian et al. 2016b). Microbial activity depends on the hydrocarbon concentration, the length of time it contaminated the soil and the soil properties (Margesin et al. 2007; Lauber et al. 2008). Different microorganisms can be found in aged contaminated soil when compared to that of the freshly contaminated soil (Militon et al. 2010; Abbasian et al. 2016b). Changes in soil conditions may influence the composition and diversity of soil microbial community (Aleer et al. 2014; Liang et al. 2014).

At the phylum level the change occurred in the phyla Bacteroidetes, Proteobacteria and Acidobacteria as it has already been reported (Militon et al. 2010; Sutton et al. 2013; Abbasian et al. 2016a). Regarding Acidobacteria, this group of bacteria is phylogenetically diverse, and widespread in soils with many members, notably the Gp groups that are largely uncultured (Kuske et al. 1997; Naether et al. 2012). In the case of the PAH amended soil there were clones from the Gp groups 3, 4, 6 and 10, with Gp6 being the most dominant, that accounted for all of the increase in this phylum, which expanded from 0.4% to 5.1% of the total clones. However, due to the lack of cultivation of these groups their relation to PAH degradation is unclear. The obtaining of the uncultured groups is very common in the microbial diversity study. Large amounts of these bacteria were also found in the similarly contaminated soil in various geographical locations (Sutton et al. 2013; Cury et al. 2015). In contrast, the Bacteroidetes saw a large drop in numbers from 17% of the total 16S rRNA gene clones to 4% and this occurred across all classes identified. In addition, with regard to those clones that were identified as Bacteroidetes in the PAH amended soil, there was no indication of a selection for PAH degraders within this phylum.

Proteobacteria were identified as the main phylum present in hydrocarbon-contaminated soil samples and these microorganisms had an important role in the natural attenuation of hydrocarbons (Sutton et al. 2013). Among these phyla, bacteria belonging to Alpha-, Beta-, and Gammaproteobacteria degrade aliphatic and aromatic compounds into simpler forms (Kostka et al. 2011). Upon contamination with petroleum hydrocarbons a shif of the relative abundance of Proteobacteria is often noted (Sutton et al. 2013). In this work, the comparison of the microbial communities in the phylum Proteobacteria reveals a statistically significant increase in the Alphaproteobacteria, a slightly higher percentage of Gammaproteobacteria clones and an effectively equivalent number of Betaproteobacteria clones in the PAH amended soil vs. the unamended control. The Deltaproteobacteria were a tiny fraction (0.4%) of the clones from the unamended control on day 22 and were undetected in the clones form the PAH amended soil.

Within the Alphaproteobacteria the most significant difference was an almost 2-fold increase in the number of clones from the order Sphingomonadales (4% vs. 7.6%), largely in the families Erythrobacteraceae (1.6% vs. 2.5%) and Sphingomonadaceae (1.6% vs. 4.2%). In addition, there was a large increase in the unclassified Alphaproteobacteria from 1.6% to 4.2% of the total clones. The family Sphingomonadaceae contains many PAH degraders notably in the genera *Sphingomonas* and *Sphingopyxis* (Ho et al. 2000; Kertesz and Kawasaki 2010; Shokrollahzadeh et al. 2012). In this case the genus *Sphingopyxis* increased from 1.2% to 2.5% of the total population and the genus *Sphingomonas* increased from 0 to 1% of the total clones. The genus *Brevundimonas* in the family Caulobacteraceae remained relatively constant with only slight increase in the percentage of clones. *Brevundimonas*, which represented 5% of the population in the soil enrichment, have been identified frequently in PAH contaminated soils and this genus is known to contain various PAH degrading species (Seo et al. 2007; Phillips et al. 2008). There was also an increase in the order Rhizobiales (6.5% vs. 9.3%). Approximately half of the identified Rhizobiales in the PAH amended microcosm could not be identified at the genus level and the remainder were identified as members of the genera *Microvirga*, *Bosea*, and *Devosia*. Interestingly, *Bosea* represented 5% of the population in the soil enrichment culture and *Bosea* sp. have been isolated from PAH contaminated soil but did not demonstrate the ability to degrade PAHs themselves suggesting they may metabolize PAH degradation products produced by other microorganisms (Seo et al. 2007). *Devosia* been identified in PAH contaminated microcosms, however, to our knowledge, *Microvirga* has not associated with PAH degradation (Guazzaroni et al. 2013).

Although the total number of Betaproteobacteria clones were approximately equivalent in the unamended control versus the PAH amended soil there were differences in the distribution of identified genera; however, these differences were not scored as statistically significant at the $p < 0.01$ level. In particular, in the PAH amended soil, there was a 2 fold increase in the number of clones from the family Alcaligenaceae of 3.2% to 6.8% of the total clones. The increase in the Alcaligenaceae occurred within the genera *Achromobacter* (0.4 vs. 0.8%), *Bordetella* (0.4% vs. 2.5%) and *Pusillimonas* 1.6% vs. 3.4%), and all of these groups contain frequently identified PAH degraders (Eriksson et al. 2003; Seo et al. 2007; Hilyard et al. 2008, Prince et al. 2010). Interestingly, *Achromobacter* and *Bordetella* were the two genera identified in the Betaproteobacteria in the soil enrichment culture. The complete genome of *Pusillimonas* sp. T7-7, isolated from the benthal mud of a petroleum-contaminated site in Bohai Sea, China, has been sequenced (Cao et al. 2011), and a variety of aromatic degradation genes have been identified, including those involved in metabolism of methylnaphthalene, phenanthrene and anthracene that can be found in the KEGG PATHWAY Database (http://www.genome.jp/kegg/).

In the Gammaproteobacteria, a large decline occurred across all identified orders with the exception of Pseudomonadales, which remained equivalent (1.6% vs. 1.7%) with the only identified genus being

Table I
Putative PAH degrading genera identified in the soil enrichment and soil microcosms.

Phylum	Soil Enrichment – Day 22	PAH Amended – Day 8	Augmented – Day 8	PAH Amended – Day 22	Bioaugmented – Day 22
Bacteriodetes	*Sediminibacterium*				*Flavobacterium*
					Arenibacter
Alphaproteobacteria	*Azospirillum*				
	Bosea			*Bosea*	
	Brevundimonas			*Brevundimonas*	
				Devosia	
	Hyphomicrobium				
	Phyllobacterium				
	Sphingomonas			*Sphingomonas*	*Sphingomonas*
				Sphingopyxis	
Betaproteobacteria	*Achromobacter*			*Achromobacter*	
	Bordetella			*Bordetella*	
		Pusillimonas		*Pusillimonas*	
Gammaproteobacteria	*Pseudomonas*			*Pseudomonas*	
	Pseudoxanthomonas			*Pseudoxanthomonas*	*Pseudoxanthomonas*
	Steroidobacter				*Steroidobacter*
Firmicutes		*Bacillus*	*Bacillus*	*Bacillus*	*Bacillus*
		Paenibacillus			
Candidatus Saccharibacteria		*Saccharibacteria genera incertae sedis*	*Saccharibacteria genera incertae sedis*	*Saccharibacteria genera incertae sedis*	*Saccharibacteria genera incertae sedis*

Pseudomonas. In addition, although members of the Gammaproteobacteria order Xanthomonadales declined from 9 to 5.1% of the total population, the genus *Pseudoxanthomonas* within the order Xanthomonadales increased form 0% to 1.7% and members of this group are known to be PAH degraders (Prince et al. 2010; Patel et al. 2012).

With regard to the Firmicutes, which contain the genus *Bacillus*, both the unamended control and the PAH amended soil had an effectively equivalent distribution of clones, 29% and 28%, respectively. These clones came almost entirely from the families *Clostridia* and *Bacilliaceae*, with both families representing approximately 50% of the total clones both sets of microcosms. Within the family *Bacilliaceae* both sets of microcosms were dominated by the genus *Bacillus* and unclassified members of the family *Bacillaceae*, which contains the genus *Bacillus*. Members of the genus *Bacillus* have frequently been identified as PAH degraders in soils.

Comparison of the microbial community structure between the PAH amended and bioaugmented microcosms. On day 8, which represents the onset of PAH degradation, the identified microbial communities were statistically equivalent between the PAH amended (316 clones) and bioaugmented (338 clones) microcosms with the exception of the genera *Saccharibacteria* genera incertae sedis (10% vs. 18%) within the phylum Candidatus Saccharibacteria (previously referred to as Candidate Division TM7), *Paenibacillus* (3% vs. 0.3%), within the phylum Firmicutes, and *Pusillimonas* (3% vs. 0.3%), within the Betaproteobacteria. *Paenibacillus* and *Pusillimonas* have been previously identified as PAH degraders and *Saccharibacteria* genera incertae sedis, the only genus of the candidate phylum *Candidatus Saccharibacteria* has recently been associated with benzene degradation (Xie et al. 2011), and these bacteria are ubiquitous phylum found in soils, sediments and wastewater (Ferrari et al. 2014).

Comparison of the PAH amended to the bioaugmented microcosms at the phylum level (Fig. 3) on day 22 shows a statistically significant lower abundance of Proteobacteria clones in the bioaugmented soil, 42.8% vs. 24.1% of the total clones, respectively. In contrast, a slight overall increase was observed within the Proteobacteria in the PAH amended soil in comparison to the unamended control.

Comparison of the identified Proteobacteria from the bioaugmented soil to the PAH amended soil reveals a statistically significant reduction (50%) in Alphaproteobacteria and an almost complete reduction in the Betaproteobacteria (10% vs. 0.5%). Moreover, these population reductions included virtually all of the putative aromatic degraders that increased in the soil amended with PAHs alone, with the exception of an increase in the PAH degrading Alphaproteobacteria

genus *Sphingomonas* (0.8% to 1.6% of the total), suggesting that the added bacterial inoculum in some way suppressed this population in the later stages of PAH degradation. The Gamaproteobacteria remained roughly constant both in numbers and the distribution of genera with the exception that the genus *Pseudomonas* was not detected in the bioaugmented microcosms while the genus *Steroidobacter* was (2% vs. 0%).

In contrast to the Proteobacteria, clones identified as members of the phyla Actinobacteria were considerably more abundant comprising 9% of the total clones in the PAH amended and bioaugmented soil versus 3.4% for the PAH amended. Although the levels of Actinobacteria were significantly higher in the bioaugmented microcosms compared to the PAH amended alone, they largely mirrored those of the unamended control day 22 and there was no indication of a selection for PAH degraders.

Given the majority of the bacteria used for bioaugmentation were *Bacillus* species, it was of particular interest to see if these organisms persisted and may then have played a role in PAH degradation. The percentage of clones identified as members of the phylum Firmicutes are very similar between all three microcosms (Fig. 3) with all microcosms being dominated by the classes Bacilli and Clostridia, and notably in the genus *Bacillus*, which represented approximately 4–5% of the total in all cases. Although the genus *Bacillus* contains many well documented PAH degraders, the abundance of this genus in all 3 types of microcosms makes it unclear as to what extent they played a role in PAH degradation. However, 2% of the clones from bioaugmented microcosm on day 8 were identical in sequence to 2 of the *Bacillus* strains used for bioaugmentation suggesting these strains persisted in the microcosms up to this point. On day 22 these strains were not detected in the bioaugmented microcosm and none of the 16S sequences from the added organisms were detected at either time point in the microcosm only receiving PAHs. Nonetheless, 16S rRNA gene sequence identity does not infer secondary metabolic attributes such as PAH degradation and it is possible the matching clones identified in the bioaugmented microcosms were not from the inoculated strains.

Moreover, the fact that no matching sequences were identified on day 22 in the bioaugmented microcosm suggest that bioaugmentation did not result in the persistence of the added organisms throughout the PAH degradation period. The adaptation of the introduced microorganisms to the environmental conditions and maintenance of high metabolic activity seem to be an important aspect in the bioremediation. However, multitude of the biotic and abiotic factors may affect the success of this process (Szczepaniak et al. 2016). Among the biotic factors, the competition between indigenous microorganisms and those introduced via bioaugmentation is mentioned most frequently (Mrozik and Piotrowska-Seget 2010). Other factors like the insufficient abundance, predation and other antagonistic activities (i.e. secretion of antibiotics) also can be the causes of failure (Thompson et al. 2005; Szulc et al. 2014); hence, they may also be the most plausible causes for no persistence of bioaugmentation during this study.

On the other hand, the question then remains as to which members of the microbial community in the PAH bioaugmented are likely to be the dominant enriched PAH degraders. The most likely candidates are to be found in the clones identified as members of the phylum Bacteroidetes which had statistically significant higher percentage of the total clones (4.2% vs. 18.1) in the bioaugmented soil relative to the PAH amended soil. However, the percentage of total Bacteroidetes is effectively equivalent to that of unamended control on day 22 and as such would not suggest a significant difference between the two soils. Nonetheless, comparison of the Bacteroidetes clones at the family level reveals significant differences between the three soils as shown in Fig. 4.

As can be seen, the distribution of the Bacteroidetes classes between the 3 soils is quite different. Both the PAH amended and PAH amended and bioaugmented soils saw relatively equivalent reduction in the percentage Sphingobacteria relative to the unamended control. The dominant groups in the Sphingobacteria in the unamended soil were classified in the genus *Pedobacter* in the family Sphingobacteriaceae and also in the genus *Chitinophaga* and uncharacterized members of the family Chitinophagaceae, and both of these groups were greatly reduced in the PAH amended and PAH amended and bioaugmented soils. However, a statistically significant difference in the PAH amended and bioaugmented soil was observed in the percentage of identified clones in the class Flavobacteria, which had a large increase from 1.6% to 8% of the total clones relative to the unamended control, and was completely undetected in the PAH amended soil. The only identified Flavobacteria genus in the PAH amended and bioaugmented and unamended control was *Flavobacterium* in the family Flavobacteriaceae, which are well documented to contain a diverse range of PAH degrading capabilities (Widada et al. 2002) and have also been isolated from oil contaminated soil (Haudhary and Kim 2018). The majority of the Flavobacteriaceae clones were not able to be classified at the genus level at high confidence in the PAH amended and bioaugmented soil on day 22, which is not at all uncommon in soil samples and indicates that isolates that are very closely related to the identified clones have not yet been cultivated and characterized. Nonetheless, the majority

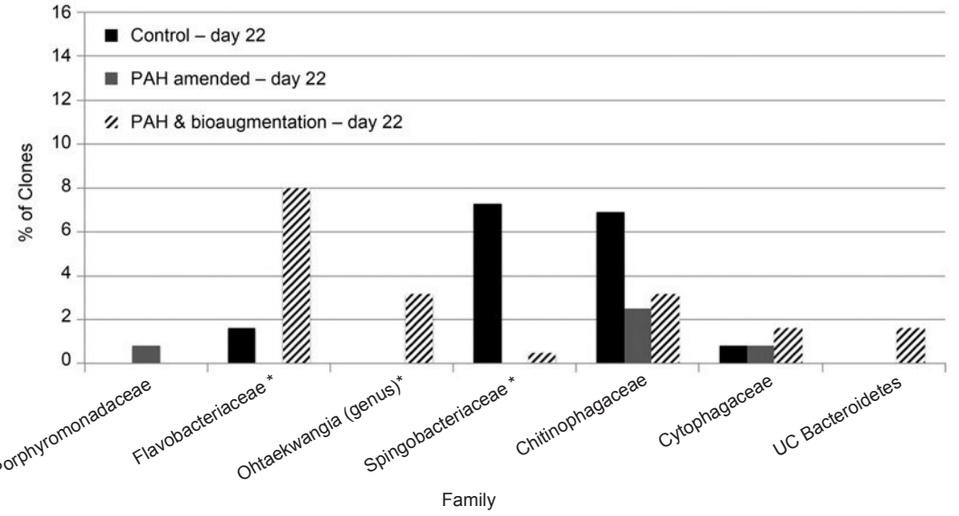

Fig. 4. Comparison of the percentage of Bacteroidetes clones at the family level (and genus level for *Ohtaekwangia*) between the unamended control, PAH amended and PAH amended and bioaugmented soils on day 22.

of the unclassified clones appeared to be most closely related to the genus *Arenibacter* (86%), an isolate of which has recently be identified as aromatic hydrocarbon-degrading bacterium and *Arenibacter* have also been previously isolated from oil contaminated sites (Kadali et al. 2012; Gutierrez et al. 2014). The remaining unclassified clones were identified as *Kriegella* and *Sediminibacter* (the only genus identified in the phylum Bacteroidetes in the soil enrichment culture).

In addition, *Ohtaekwangia*, a newly discovered genus within the phylum Bacteroidetes isolated from a sand sample collected from the west coast of the Korean peninsula using low-nutrient media (Yoon et al. 2011), increased from 0% to 3.2% of the total clones. To our knowledge it is not yet known whether this genus is associated with PAH degradation. However, given their previous association with PAH degradation it is reasonable to infer that the unclassified Flavobacteriaceae are indeed PAH degraders and it is also suggestive that *Ohtaekwangia* may also be PAH degraders as well. Further characterization of these and other unclassified clones is essential to a deeper understanding of their PAH degrading abilities.

Conclusions

A pristine soil was evaluated for its degradation potential and changes in the microbial community after exposure to the PAHs naphthalene, phenanthrene, anthracene and pyrene when used as an inoculum in a PAH enrichment in liquid mineral medium and in microcosms receiving either PAHs alone or the PAHs with bioaugmentation with PAH degrading isolates. The microbial community obtained in the enrichment culture degraded all the PAHs and consisted entirely of members of two phyla, Proteobacteria and Bacteroidetes. The soil enrichment culture possessed low microbial diversity with only the phyla Bacteroidetes and Proteobacteria represented. The Bacteroidetes represented a small fraction relative to the Proteobacteria and consisted only of *Sediminibacter* and closely related unclassified bacteria, while the Proteobacteria were represented by Alpha, Beta and Gamaproteobacteria.

The soil microcosms receiving only PAHs and receiving PAHs and bioaugmentation showed effectively equal rates of degradation of all of the PAHs with complete degradation occurring by day 30 demonstrating that microbial population of the pristine soil readily degraded these compounds and that bioaugmentation did not improve degradation rates.

At the onset of PAH degradation (day 8) the putative PAH degraders microcosms (PAH amended and bioaugmented) were similar with the exception of a significant population of the genus *Pusillimonas* in the PAH amended microcosms (3% of the total). Identical sequence to two of the bioaugmention inoculum strains were identified in the bioaugmented microcosms, however, these sequences were not detected at the later stages of PAH degradation (day 22) indicating the added PAH degrading isolates did not persist throughout the degradation period. On day 22 the PAH amended soil showed increases in putative PAH degraders primarily in the Alphaproteobacteria genera *Sphingomonas* and *Sphingopyxis*, and members of the order Rhizobiales and in the Betaproteobacteria in the family Alcaligenaceae within the genera *Achromobacter*, *Bordetella*, *Pusillimonas*, all of which contain

well documented PAH degraders. In marked contrast, the increase in putative PAH degraders in the PAH amended and bioaugmented soil occurred in the Bacteroidetes family Flavobacteriaceae, and were classified as members of the genus *Flavobacterium* or were unclassified Flavobacteriaceae, and also the newly identified genus *Ohtaekwangia*. In addition, there was no evidence for a persistent increase in clones from the consortia of PAH degrading bacteria used for bioaugmentation.

These results indicate that soils can contain a diversity of microbial taxa that can degrade PAHs with equivalent efficiency, however, which groups become most dominant is flexible and dependent not only the PAH composition.

Acknowledgments

We thank the Coordinator for the Improvement of Personnel in Higher Education (CAPES, Brazil), the National Council for the Development of Science and Technology (CNPq), and the Foundation for the Support of Science in São Paulo State (FAPESP) for financial support.

Literature

Abbasian F, Lockington R, Megharaj M, Naidu R. 2016a. The biodiversity changes in the microbial population of soils contaminated with crude oil. Curr Microbiol. 72:663–670.

Abbasian F, Palanisami T, Megharaj M, Naidu R, Lockington R, Ramadass R. 2016b. Microbial diversity and hydrocarbon degrading gene capacity of a crude oil field soil as determined by metagenomics analysis. Biotechnol. Prog. 32:638–648.

Aleer S, Adetutu EM, Weber J, Ball AS, Juhasz AL. 2014. Potential impact of soil microbial heterogeneity on the persistence of hydrocarbons in contaminated subsurface soils J Environ Manag. 136:27–36.

Bento FM, Camargo FA, Okeke BC, Frankenberger WT. 2005. Comparative bioremediation of soils contaminated with diesel oil by natural attenuation, biostimulation and bioaugmentation. Bioresour Technol. 96:1049–1055.

Brown SD, Utturkar SM, Klingeman DM, Johnson CM, Martin SL, Land ML, Lu TY, Schadt CW, Doktycz MJ, Pelletier DA. 2012. Twenty-one genome sequences from Pseudomonas species and 19 genome sequences from diverse bacteria isolated from the rhizosphere and endosphere of *Populus deltoides*. J Bacteriol. 194:5991–5993.

Cao BY, Ma T, Ren Y, Li GQ, Li P, Guo X, Ding P, Feng L. 2011. Complete genome sequence of *Pusillimonas* sp. T7-7, a cold-tolerant diesel oil-degrading bacterium isolated from the Bohai Sea in China. J Bacteriol. 193:4021–4022.

Chaudhary DK, Kim J. 2018. *Flavobacterium naphthae* sp. nov., isolated from oil-contaminated soil. Int J Syst Evol Microbiol. 68:305–309.

Cole JR, Chai B, Farris RJ, Wang Q, Kulam SA, McGarrell DM, Garrity GM, Tiedje JM. 2005. The Ribosomal Database Project (RDP-II), sequences and tools for high-throughput rRNA analysis. Nucleic Acids Res. 33:D294–D296.

Cole JR, Wang Q, Cardenas E, Fish J, Chai B, Farris RJ, Kulam-Syed-Mohideen AS, McGarrell DM, Marsh T, Garrity GM, Tiedje JM. 2009. The Ribosomal Database Project, improved alignments and new tools for rRNA analysis. Nucleic Acids Res. 37:D141–145.

Curry JC, Jurelevicius DA, Villena HDM, Jesus HE, Peixoto RS, Schaefer CEGR, Bícego MC, Seldin L, Rosado AS. 2015. Microbial diversity and hydrocarbon depletion in low and high diesel-polluted soil samples from Keller Peninsula, South Shetland Islands. Antarct Sci. 27:263–273.

Eisler R. 1987. Polycyclic aromatic hydrocarbon hazards to fish, wildlife and invertebrates, a synoptic review. Contaminant Hazard Reviews. Report 11: Biological Report 85(1.11). Laurel, MD (USA): U.S. Department of the Interior, Fish and Wildlife Service.

Eriksson M, Sodersten E, Yu Z, Dalhammar G, Mohn WW. 2003. Degradation of polycyclic aromatic hydrocarbons at low temperature under aerobic and nitrate-reducing conditions in enrichment cultures from northern soils. Appl Environ Microbiol. 69:275–284.

Ferrari B, Winsley T, Ji M, Neilan B. 2014. Insights into the distribution and abundance of the ubiquitous candidatus Saccharibacteria phylum following tag pyrosequencing. Sci Rep. 4:3957.

Guazzaroni ME, Herbst FA, Lores I, Tamames J, Peláez AI, López-Cortés N, Alcaide M, Del Pozo MV, Vieites JM, von Bergen M, et al. 2013. Metaproteogenomic insights beyond bacterial response to naphthalene exposure and bio-stimulation. ISME J. 7:122–136.

Gutierrez T, Rhodes G, Mishamandani S, Berry D, Whitman WB, Nichols PD, Semple KT, Aitken MD. 2014. Polycyclic aromatic hydrocarbon degradation of phytoplankton-associated *Arenibacter* spp. and description of *Arenibacter algicola* sp. nov., an aromatic hydrocarbon-degrading bacterium. Appl Environ Microbiol. 80(2): 618–628.

Haritash AK, Kaushik CP. 2009. Biodegradation aspects of polycyclic aromatic hydrocarbons (PAHs): A review. J Hazard Mater. 169:1–15.

Hilyard EJ, Jones-Meehan JM, Spargo BJ, Hill RT. 2008. Enrichment, isolation and phylogenetic identification of polycyclic aromatic hydrocarbon-degrading bacteria from Elizabeth River sediments. Appl Environ Microbiol. 74:1176–1182.

Ho Y, Jackson MM, Yang Y, Mueller JG, Pritchard PH. 2000. Characterization of fluoranthene- and pyrene-degrading bacteria isolated from PAH-contaminated soils and sediments and comparison of several *Sphingomonas* spp. J Ind Microbiol Biotech. 24:100–112.

Janssen PH. 2006. Identifying the dominant soil bacterial taxa in libraries of 16S rRNA and 16S rRNA genes. Appl Environ Microbiol. 72:1719–1728.

Juhasz AL, Naidu R. 2000. Bioremediation of high molecular weight polycyclic aromatic hydrocarbons, a review of the microbial degradation of benzo[a]pyrene. Int Biodeterior Biodegradation. 45:57–88.

Kadali KK, Simons KL, Skuza PP, Moore RB, Ball AS. 2012. A complementary approach to identifying and assessing the remediation potential of hydrocarbonoclastic bacteria. J Microbiol Methods. 88:348–355.

Kertesz MA, Kawasaki A. 2010. Hydrocarbon-degrading Sphingomonads, *Sphingomonas*, *Sphingobium*, *Novosphingobium*, and *Sphingopyxis*. In: Timmis KN, McGenity T, Meer JR, Lorenzo V, editors. Handbook of hydrocarbon and lipid microbiology. Berlin Heidelberg (Germany): Springer. p. 1693–1705.

Khan MAI, Biswas B, Smith E, Mahmud SA, Hasan NA, Khan MAW, Naidu R, Megharaj M. 2018. Microbial diversity changes with rhizosphere and hydrocarbons in contrasting soils. Ecotoxicol Environ Saf. 156:434–442.

Kostka JE, Prakash O, Overholt WA, Green SJ. Freyer G, Canion A, Delgardio J, Norton N, Hazen TC, Huettel M. 2011. Hydrocarbon-degrading bacteria and the bacterial community response in Gulf of Mexico beach sands impacted by the deepwater horizon oil spill. Appl Environ Microbiol. 77:7962–7974.

Kuske CR, Barns SM, Busch JD. 1997. Diverse uncultivated bacterial groups from soils of the arid southwestern United States those

are present in many geographic regions. Appl Environ Microbiol. 63:3614–3621.

Lane DJ. 1991. 16S/23S rRNA sequencing. In: Stackebrandt E, Goodfellow M, editors. Nucleic acid techniques in bacterial systematics. New York (USA): John Wiley and Sons. p. 115–175.

Lauber CL, Strickland MS, Bradford MA, Fierer N. 2008. The influence of soil properties on the structure of bacterial and fungal communities across land-use types. Soil Biol Biochem. 40:2407–2415.

Liang Y, Zhao H, Zhang X, Zhou J, Li G. 2014. Contrasting microbial functional genes in two distinct saline-alkali and slightly acidic oil-contaminated sites. Sci Total Environ. 487:272–278.

Lindstrom JE, Prince RC, Clark JC, Grossman MJ, Yeager TR, Braddock JF, Brown, EJ. 1991. Microbial populations and hydrocarbon biodegradation potentials in fertilized shoreline sediments affected by the T/V Exxon Valdez oil spill. Appl Environ Microbiol. 57:2514–2522.

Margesin R, Hämmerle M, Tscherko D. 2007. Microbial activity and community composition during bioremediation of diesel-oil-contaminated soil: effects of hydrocarbon concentration, fertilizers, and incubation time. Microbiol Ecol. 53:259–269.

Megharaj M, Ramakrishnan B, Venkateswarlu K, Sethunathan N, Naidu R. 2011. Bioremediation approaches for organic pollutants: a critical perspective. Environ Int. 37:1362–1375.

Militon C, Boucher D, Vachelard C, Perchet G, Barra V, Troquet J, Peyretaillade E, Peyret P. 2010. Bacterial community changes during bioremediation of aliphatic hydrocarbon-contaminated soil. FEMS Microbiol Ecol. 74:669–681.

Mrozik A, Piotrowska-Seget Z. 2010. Bioaugmentation as a strategy for cleaning up of soils contaminated with aromatic compounds. Microbiol Res. 165:363–375.

Naether A, Foesel BU, Naegele V, Wüst PK, Weinert J, Bonkowski M, Alt F, Oelmann Y, Polle A, Lohaus G, et al. 2012. Environmental factors affect Acidobacterial communities below the subgroup level in grassland and forest soils. Appl Environ Microbiol. 78:7398–7406.

Patel V, Cheturvedula S, Madamwar D. 2012. Phenanthrene degradation by *Pseudoxanthomonas* sp. DMVP2 isolated from hydrocarbon contaminated sediment of Amlakhadi canal, Gujarat, India. J Hazard Mater. 201–202:43–51.

Petry T, Schmid P, Schlatter C. 1996. The use of toxic equivalency factors in assessing occupational and environmental health risk associated with exposure to airborne mixtures of polycyclic aromatic hydrocarbons (PAHs). Chemosphere. 32:639–648.

Philp JC, Atlas RM. 2005. Bioremediation of contaminated soil and aquifers. In: Atlas R M, Jim CP, editors. Bioremediation: Applied Microbial Solution for Real – World Environmental Clean Up. Washington DC (USA): ASM Press. p. 139.

Phillips LA, Germida JJ, Farrell RE, Greer CW. 2008. Hydrocarbon degradation potential and activity of endophytic bacteria associated with prairie plants. Soil Biol Biochem. 40:3054–3064.

Prince R, Gramain A, McGenity T. 2010. Prokaryotic hydrocarbon degraders. In: Timmis KN, McGenity TJ, van der Meer JR, de Lorenzo V, editors. Handbook of hydrocarbon and lipid microbiology. Berlin (Germany): Springer. p. 1669–1692.

Rambeloarisoa E, Rontani JF, Giusti G, Duvvnjak Z, Bertrand JC. 1984. Degradation of crude oil by a mixed population of bacteria isolated from sea surface foams. Mar Biol. 83:69–81.

Samanta SK, Singh OV, Jain RK. 2002. Polycyclic aromatic hydrocarbons, environmental pollution and bioremediation. Trends Biotechnol. 20:243–248.

Sayara T, Borras E, Caminal G, Sarra M, Sanchez A. 2011. Bioremediation of PAHs-contaminated soil through composting: Influence of bioaugmentation and biostimulation on contaminant biodegradation. Int Biodeterior Biodegrad. 65:859–865.

Seo JS, Keum YS, Harada RM, Li,p QX. 2007. Isolation and characterization of bacteria capable of degrading polycyclic aromatic hydrocarbons (PAHs) and organophosphorus pesticides from PAH-contaminated soil in Hilo, Hawaii. J Sci Food Agric. 55:5383–5389.

Seo JS, Keum YS, Li QX. 2009. Bacterial degradation of aromatic compounds. Int J Environ Res Public Health. 6:278–309.

Shokrollahzadeh S, Golmohammad F, Shokouhi H. 2012. Study of *Sphingopyxis* isolates in degradation of polycyclic aromatic hydrocarbons. Chem Eng Trans. 27:55–60.

Silva IS, Costa SE, Ragagnin MC, Fonseca FA, Franciscon GDE, Grossman MJ, Durrant LR. 2009. Bioremediation of a polyaromatic hydrocarbon contaminated soil by native soil microbiota and bioaugmentation with microbial isolates and consortia. Bioresour Technol. 100:4669–4675.

Singleton DR, Richardson SD, Aitken MD. 2011. Pyrosequence analysis of bacterial communities in aerobic bioreactors treating polycyclic aromatic hydrocarbon-contaminated soil. Biodegradation 22:1061–1073.

Sutton NB, Maphosa F, Morillo JA, Al-Soud WA, Langenhoff AAM, Grotenhuis T, Smidt H. 2013. Impact of long-term diesel contamination on soil microbial community structure. Appl Environ Microbiol. 79:619–630.

Szczepaniak Z, Cyplik P, Juzwa W, Czarny J, Staninska J, Piotrowska-Cyplik A. 2015. Antibacterial effect of the *Trichoderma viride* fungi on soil microbiome during PAH's biodegradation. Int Biodeter Biodegr. 104:170–177.

Szulc A, Ambrożewicz D, Sydow M, Ławniczak Ł, Piotrowska-Cyplik A, Marecik R, Chrzanowski Ł. 2014. The influence of bioaugmentation and biosurfactant addition on bioremediation efficiency of diesel-oil contaminated soil: Feasibility during field studies. J Environ Manage. 132:121–128.

Thompson IP, Van Der Gast CJ, Ciric L, Singer AC. 2005. Bioaugmentation for bioremediation: the challenge of strain selection. Environ Microbiol. 7(7):909–915.

Tyagi M, da Fonseca MM, de Carvalho CC. 2011. Bioaugmentation and biostimulation strategies to improve the effectiveness of bioremediation processes. Biodegradation. 22:231–241.

Van Hamme JD, Singh A, Ward OP. 2003. Recent advances in petroleum microbiology. Microbiol Mol Biol Rev. 67:503–549.

Vinas M, Sabate J, Espuny MJ, Solanas AM. 2005. Bacterial community dynamics and polycyclic aromatic hydrocarbon degradation during bioremediation of heavily creosotecontaminated soil. Appl Environ Microbiol. 71:7008–7018.

Wang Q, Garrity GM, Tiedje JM, Cole JR. 2007. Naïve Bayesian Classifier for Rapid Assignment of rRNA Sequences into the New Bacterial Taxonomy. Appl Environ Microbiol. 73:5261–5267.

Widada J, Nojiri H, Kasuga K, Yoshida T, Habe H, Omori T. 2002. Molecular detection and diversity of polycyclic aromatic hydrocarbon-degrading bacteria isolated from geographically diverse sites. Appl Microbiol Biotechnol. 58:202–209.

Wilson SC, Jones KC. 1993. Bioremediation of soil contaminated with polynuclear aromatic hydrocarbons (PAHs): a review. Environ Pollut. 81:229–249.

Wolin EA, Wolin MJ, Wolfe RS. 1963. Formation of methane by bacterial extracts. J Biol Chem. 238:2882–2886.

Wyrwas B, Dymaczewski Z, Zgoła-Grześkowiak A, Szymański A, Frańska M, Kruszelnicka I, Ginter-Kramarczyk D, Cyplik P, Ławniczak Ł, Chrzanowski Ł. 2013. Biodegradation of Triton X-100 and its primary metabolites by a bacterial community isolated from activated sludge. J Environ Manage. 128:292–299.

Yoon JH, Kang SJ, Lee SY, Lee JS, Park S. 2011. *Ohtaekwangia koreensis* gen. nov., sp. nov. and *Ohtaekwangia kribbensis* sp. nov., isolated from marine sand, deep-branching members of the phylum *Bacteroidetes*. Int J Syst Evol Microbiol. 61:1066–1072.

Xie S, Sun W, Luo C, Cupples AM. 2011. Novel aerobic benzene degrading microorganisms identified in three soils by stable isotope probing. Biodegradation. 22:71–81.

Polish Journal of Microbiology
2018, Vol. 67, No 3, 377–382
https://doi.org/10.21307/pjm-2018-047

ORIGINAL PAPER

Biochemical and Molecular Characterization of a Native Haloalkalophilic Tolerant Strain from the Texcoco Lake

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Submitted 31 October 2017, revised 27 March 2018, accepted 23 June 2018

Abstract

In the last decade several new genera have been isolated in alkaline and halophile growth conditions. The studies conducted in the Texcoco Lake soils have shown a generalized microbial adaptation to the specific conditions. In this research work, morphological and phylogenetic characterization of the HN31(22) strain that was isolated from the cited soil is presented. The strain was identified as a Gram-positive halophile and alkaline tolerant bacteria from the *Nesterenkonia* genus, which uses different substrates in metabolic processes.

Key words: halophile, alkalophile, adaptation, phylogeny, *Nesterenkonia*

Introduction

Alkalophile microorganisms are defined as organisms that have an optimum growth rate in environments with alkaline pH, particularly greater than 8. A source rich in variety of these organisms are the environments with a stable alkalinity, such as saline lakes (Castillo et al. 2005). Halophile bacteria are defined as microorganisms that show a better growth in the presence of salt NaCl. Given that these microorganisms are capable of growth in different saline concentrations, the term halophile is usually reserved for those requiring a minimum of excess salt concentration, found in seawater, in general, concentrations greater than 3% (Oren, 2008). Extreme halophile bacteria are those that present an optimum growth rate in high saline concentrations, near 20% NaCl (3–4 M). These bacteria grow in hyper saline environments, such as saline lakes that present a greater range of saline concentrations than sea (Jones and Grant 2002). The soil from the Texcoco Lake is alkaline, and with electrical conductivities (EC) in saturated strata from 22 to 150 dSm^{-1} and sodium percentages from 76% to 98% (Luna-Guido et al. 2002). Several microorganisms adapted to extreme conditions have been identified from the soil of the former Texcoco Lake (Valenzuela-Encinas et al. 2008; Ruiz-Romero et al. 2013; Soto-Padilla et al. 2014). The application of molecular biological techniques to microbial ecology has shown that the cultured organisms are generally different from those that dominate in the natural environment (Oren, 2002). In addition, knowledge of their biochemical characteristics supports understanding of their metabolic processes.

In the last decade, there has been an increment in the number of newly isolated bacterial genera, which grows in alkaline and halophile conditions. The study of enzymes that aid in the metabolism of these extremophiles to operate under these conditions is of great interest (Castillo et al. 2005; Ramírez et al. 2006). Characterization and identification of native microorganisms of relevant sites, as well as specific biological and metabolic functions are closely related to protein and enzymes that work in extreme conditions. These biomolecules show unique features that can be used as models for the design and construction of proteins with new properties, which are of interest for industrial applications (Castillo et al. 2005). The aim of this study was to accomplish biochemical and phylogenetic characterization of the native halophile/alkaline tolerant bacteria from the Texcoco Lake.

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Experimental

Materials and Methods

Microorganisms. The bacterial strain HN31(22) was isolated from the soil of the Texcoco Lake, located northwest from Mexico city (Northern Latitude 19°30'52' Western Longitude 98°59'24') in the State of Mexico, Mexico (Soto-Padilla et al. 2014). The strain was cultivated and maintained in marine agar (Bacto marine agar 2216, DIFCO). Growth conditions were as described by Valenzuela-Encinas et al. (2008). Pure cultures were obtained by picking and restreaking individual colonies grown in Petri plates solidified with 2% agar at a temperature of 37°C for 48 h.

Tolerance to pH, temperature and NaCl concentration. A commercial marine medium (Bacto marine broth 2216, DIFCO) was used. The pH values from 4 to 12 were tested at a temperature of 37°C, and NaCl percentage concentrations of 1.9, 5, 10, 15 and 20 were employed. In order to determine the growth rate curves, the turbidity measurement (Cintra 10e, GBC Scientific Equipment, Australia) at 600 nm (Coronado et al. 2000; Thacker et al. 2006) to subsequently determine cell concentration using the dry biomass technique was performed. The dry weight of the bacterial cells was obtained after centrifugation of a known volume of the culture suspension and drying the pellet in an oven at 105°C for 24 hours. To establish the optimum growth temperature, the strain was incubated at various temperatures of 4, 30, 37, 40, 55°C. All experiments were done in triplicate**.**

16S rRNA gene sequencing and phylogenic analysis. DNA extraction was done for phylogenetic characterization, including amplification, sequencing and bioinformatics analysis (NCBI) (Soto-Padilla et al. 2014). The 16S rRNA gene was amplified using the universal bacterial forward and reverse primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3'), respectively. The reaction mixture (25 µl) contained 1 µl of genomic DNA; the appropriate primers (27F and 1,492R) at 0.5 µM each one; dATP, dCTP, dGTP, and dTT at 10 mM each one; 50 mM MgCl; and 1 U of Taq DNA polymerase in the PCR buffer provided by the manufacturer (Invitrogen, USA). Amplification conditions included: denaturation of the sample for 10 min at 94°C, followed by 1 min of annealing at 57°C and elongation during 2 min at 72°C for a total number of 35 cycles (Valenzuela-Encinas et al. 2008). The amplification was performed in a Touchgene Gradient thermal cycler FTGRAD2D (TECHNE DUXFORT, Cambridge, UK). Phylogenetic trees were constructed using the Neighbor-joining method; and Tamura-Nei model of distance analysis and 500 Bootstrap replications were assessed to support internal branches.

Morphologic and biochemical characterization. To determine the bacterial cell morphology, the scanning electron microscopy (SEM) was used. The cells from a 24 hours culture were collected, washed, fixed with 2% formaldehyde, and dried by dehydration using acetone and ethyl alcohol. The following biochemical tests were performed: Gram staining, oxidase activity, catalase production, urease, nitrate reduction, citrate test, MR-VP, oxidation and fermentation of sugars. Utilization of glucose, trehalose, mannitol, sucrose and fructose were evaluated according to Cowan and Steels methodology (Barrow and Feltham, 2004). Hydrolysis of Tween 80, casein, gelatin and starch were also performed using standard techniques. All experiments were done in triplicate.

Results

The HN31(22) strain was Gram-positive with cells of short roads morphology (Fig. 1) and size between 1–2 µm. Colonies showed a light yellow coloration, with bright creamy and round shape and flat borders. Growth of the bacteria was observed in NaCl at concentrations of up to 20% of (Fig. 2); the most efficient growth rate was observed when 5% NaCl was used, but the bacteria grew also when 10, 15 and up to 20% salt concentrations were employed. With this evidence, the bacterium was considered a halophile. Fig. 3 shows the growth of HN31(22) strain in the evaluated pH values, showing that an optimum growth rate was obtained at a neutral pH of 7. The bacterial multiplication was also observed at pH values of 5 to 10; however, a strong reduction was registered at pH of 11. For this reason the strain was considered as an alkaline tolerant bacteria. Table I shows the growth of the HN31(22) strain in various temperatures.

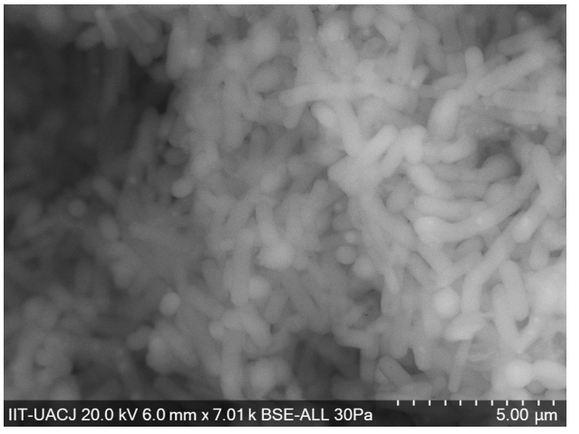

Fig. 1. A high resolution image of bacterial colonies of the HN31(22) strain taken under scanning electron microscope (SEM).

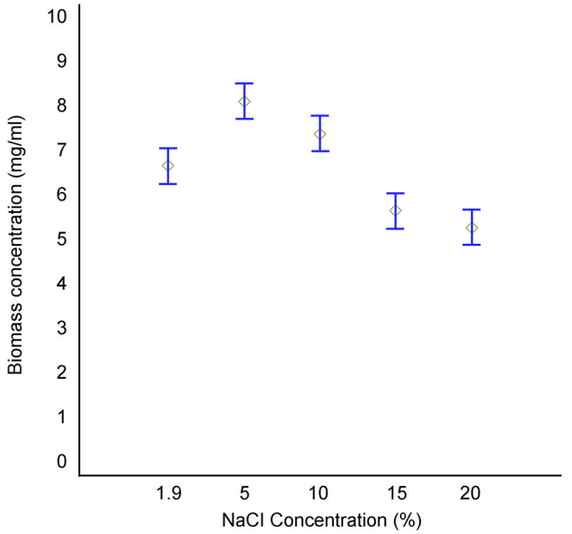

Fig. 2. The concentration of biomass (mg/ml) of the HN31(22) strain in marine medium at different NaCl concentrations (1 9–20%).

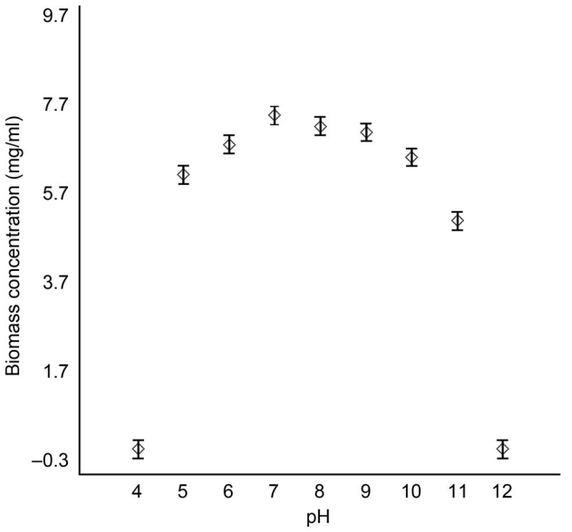

Fig. 3. The concentration of biomass (mg/ml) of the HN31(22) strain in marine medium at different pH (4–12).

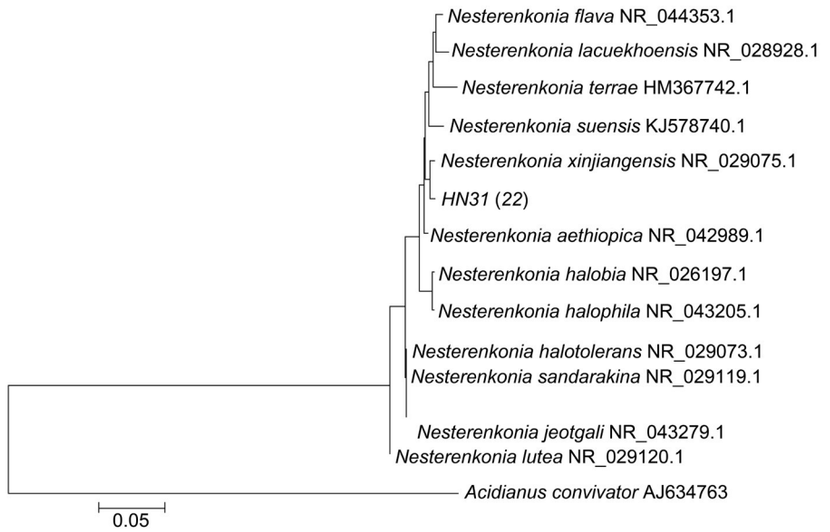

Fig. 4. Phylogenetic relationship of species of the *Nesterenkonia* genus based on the16S rDNA gene sequences.

A phylogenetic analysis was carried out and the strain isolated was classified to the genus of *Nesterenkonia* (Fig. 4) with features and shared values between 97.1% and 99.5% with other species within this genus. The evaluated biochemical traits show common characteristics with the data reported on the *Nesterenkonia* genus (Table II), in which the capacity of HN31(22) strain to use several substrates and perform different hydrolytic activities (amylase, protease, lipase) was underlined.

Table I
Growth of bacteria of the HN31(22) strain in marine agar at different temperatures (4, 30, 37, 40, 55°C).

Temperature (°C)	Growth (CFU/ml)
4	0
30	4.2×10^3
37	34×10^4
40	18×10^4
55	0

Discussion

Research studies performed on the microorganisms of the Texcoco Lake, showed that there has been a great microbial adaptation to the extreme

Table II
Comparison of phenotypic characteristics of bacteria of the HN31(22) strain with the other species of *Nesterenkonia* (1. *N. flava*, 2. *N. lacusekhoensis*, 3. *N. suensis*, 4. *N. xinjiangensis*, 5. *N. halotolerans*, 6. *N. aethiopica*, 7. *N. sandarakina*, 8. *N. lutea*, 9. *N. jeotgali*, 10. *N. halophila* (Collins et al. 2002; Li et al. 2004, 2005, 2008; Delgado et al. 2006; Yoon et al. 2006; Luo et al. 2008; Govender et al. 2013).

Characteristic	HN31(22)	1	2	3	4	5	6	7	8	9	10
Morphology	Short roads	Short roads	Short roads	Short roads	Short roads	Cocci	Short roads	Cocci	Cocci	Cocci	Cocci
Tincion	HN31(22)	Gram +	Gram +	Gram +	Gram +	Gram +	Gram +	Gram +	Gram +	Gram +	Gram +
Colony pigmentation	Yellow	Yellow	Yellow	Yellow	Yellow	Orange/ Yellow	Yellow	Orange/ Yellow	Yellow	Yellow	White ivory
Temperature (°C)	30–40	40–42	8.5–42	35–37	20–40	4–40	25–40	4–36	4–36	4–36	NR
pH	5–11	8–12	7.5–9.5	7–11	7–12	7–9	7–11	5–12	6.5–10	6–8.5	6–10.5
NaCl (%)	0–20	0–10	0–15	0–18	0–25	0–25	3–12	1–15	0–20	0–16	0.5–30
Catalase	+	+	NR	+	NR	NR	+	NR	NR	NR	NR
Oxidase	–	–	–	–	–	–	+	–	–	–	–
Urease	–	NR	NR	NR	+	+	–	–	–	–	NR
Nitrate reduction	–	–	–	+	–	–	NR	–	+	–	+
Citrate test	+	–	W	–	NR	NR	–	NR	NR	NR	NR
Voges-Proskauer	–	–	–	–	NR	NR	–	–	–	+	NR
Acid produced from:											
Galactose	–	–	–	–	–	–	–	+	+	+	NR
Trehalose	+	–	+	–	–	–	–	W	+	W	–
Mannitol	+	–	–	–	–	–	–	+	+	+	NR
Sucrose	+	NR	NR	–	–	–	–	NR	NR	NR	–
Fructose	+	NR	NR	+	–	–	+	NR	NR	NR	NR
Utilization of:											
Glucose	+	+	NR	+	+	+	+	+	W	+	+
Trehalose	+	–	+	–	–	–	–	+	–	+	NR
Fructose	–	+	+	+	+	+	+	+	+	+	+
Mannose	+	+	+	+	+	+	–	+	+	W	+
Sucrose	+	+	NR	+	+	+	W	+	+	+	NR
Maltose	+	+	NR	–	+	+	NR	+	+	+	NR
Hydrolysis of:											
Starch	+	+	–	+	–	–	+	–	+	–	–
Gelatin	+	+	–	NR	+	+	+	+	–	–	NR
Tween 80	+	+	–	+	NR	NR	NR	–	–	–	–
Casein	+	NR	NR	–	+	–	+	–	+	NR	–

(+) Positive, (–) negative, (w) weak reaction, (NR) unreported.

environmental conditions (Luna-Guido and Dendooven 2001; Soto-Padilla et al. 2014; Valenzuela-Encinas et al. 2008). Soto-Padilla et al. (2014) reported the presence of the genera with halophile and alkaline characteristics, such as: *Salinicoccus*, *Kocuria*, *Micrococcus* and *Nesterenkonia*. Morphologic characterization has been done for a new strain of *Nesterenkonia* genus, and the existing relation with the bacteria reported (Table II) concurs with those of Gram-positive bacteria, yellow colonies, and growth at pH values of alkaline range, and in high salt concentrations (NaCl). Ramírez et al. (2006) proposed bacterial classification as a function of the required salt needed for growth; and based on the data from Fig. 2 and Table II, the HN31(22) strain has been classified as a halophile. The applications of this type of bacteria in biotechnology can be divided into a number of categories; for example, food, plastics and pharmaceutical industry and bioremediation (Castillo-Carvajal and Barragán-Huerta 2011). The halophile tolerance of enzymes can be exploited when enzymatic activities are required in low water activity, such as those found in the presence of high salt concentrations. The interesting product applications for some organic osmotic stabilizers, produced by halophiles, have also been found. Several halophile microorganisms produce valuable compounds (enzymes, biopolymers), some of

those are unique and cannot be found in other type of microorganism (Oren 2002). Oarga (2009) defined an alkaline microorganism, as one with an optimum growth rate above two units above the neutral pH, and that requires high pH values for its growth; each organism has a defined pH interval, in which growth is possible (optimum pH). Most microorganism grow at a pH range between 5–9, only a few species can grow in pH values above 10 and below 2. It has been known that very few microorganisms are capable to grow in pH values close to 0 (Castillo et al. 2005).

The *Nesterenkonia* genus belongs to the Micrococcaceae family; it was proposed by Stackebrandt et al. (1995), who performed taxonomic dissection of the genus Micrococcus. Initially only the *Nesterenkonia halobia*, originally classified as *Micrococus halobius* (Onishi and Kamekura 1972), isolated from unrefined salt from Noda in Japan was included in this genus. There has been already 14 families reported, all of which represent extremophile microorganism species (Bakhtiar et al. 2003; Bakhtiar et al. 2005; Amoozegar et al. 2007). Species belonging to the *Nesterenkonia* genus have been applied for the production of certain enzymes and metabolites of interest given their biochemical characteristics (Rivadeneyra et al. 2000; Govender et al. 2009; Shafiei et al. 2010, 2012; Nel et al. 2011). The HN31(22) strain isolated from the Texcoco Lake, was classified as a Gram-positive, halophile/alkaline tolerant microorganism from the *Nesterenkonia* genus that uses starch, gelatin, casein and Tween 80 as its metabolic substrates .

Acknowledgment

This work was funded by Conacyt (Mexico).

The authors thank the support granted by Program for the promotion and support of research projects (PROFAPI-ITSON 2018-1076 and 2018-1169).

Literature

Amoozegar MA, Ghasemi A, Razavi MR, Naddaf S. 2007. Evaluation of hexavalent chromium reduction by chromate-resistant moderately halophile, *Nesterenkonia* sp. strain MF2. Process Biochem. 42:1475–1479.

Bakhtiar S, Andersson MM, Gessesse A, Mattiasson B, Hatti-Kaul R. 2003. Stability characteristics of a calcium-independent alkaline protease from *Nesterenkonia* sp. Enzyme Microb Tech. 32:525–531.

Bakhtiar S, RJ Estiveira, Hatti-Kaul R. 2005. Substrate specificity of alkaline protease from alkaliphilic feather-degrading *Nesterenkonia* sp. AL20. Enzyme Microb Tech. 37:534–540.

Barrow G, Feltham RKA. 2004. Cowan and Steel's manual for the identification of medical bacteria. Cambridge (United Kingdom): Cambridge University Press.

Castillo F, Roldán MD, Blasco R, Huertas MJ, Caballero FJ, Moreno-Vivián C, Luque-Romero MM. 2005. Biotecnología ambiental. Madrid (España): Tébar, S.L.

Castillo-Carvajal L, Barragán-Huerta BE. 2011. Aplicaciones biotecnológicas de microorganismos halófilos. Revista Sistemas Ambientales. 4:45–54.

Collins M, Lawson P, Labrenz M, Tindall B, Weiss N, Hirsch O. 2002. *Nesterenkonia lacusekhoensis* sp. nov., isolated from hypersaline Ekho Lake, East Antarctica, and emended descripction of the genus Nesterenkonia. Int J Syst Evol Microbiol. 52:1145–1150.

Coronado MJ, Vargas C, Hofemeister J, Ventosa A, Nieto JJ. 2000. Production and biochemical characterization of an α-amylase from the moderate halophile *Halomonas meridiana*. FEMS Microbiol Lett. 183:67–71.

Delgado O, Quillaguamán J, Bakhtiar S, Mattiasson B, Gessesse A, Hatti-Kaul R. 2006. *Nesterenkonia aethiopica* sp. nov., an alkaliphilic, moderate halophile isolated from an Ethiopian soda lake. Int J Syst Evol Microbiol. 56:1229–1232.

Govender L, Naidoo L, Setati ME. 2009. Isolation of hydrolase producing bacteria from Sua pan solar salterns and the production of endo-1,4-bxylanase from a newly isolated haloalkaliphilic *Nesterenkonia* sp. Afr J Biotechnol. 8:5458–5466.

Govender L, Naidoo L, Setati ME. 2013. *Nesterenkonia suensis* sp. nov., a haloalkaliphilic actinobacterium isolated from a salt pan. Int J Syst Evol Micr. 63:41–46.

Jones BE, Grant WD. 2002. Haloalkaliphilic microorganisms. U.S. Patent No. 6,420,147. Washington, DC (USA): U.S. Patent and Trademark Office.

Li WJ, Chen HH, Xu P, Zhang YQ, Schumann P, Tang SK, Xu LH, Jiang CL. 2004. *Yania halotolerans* gen. nov., sp. nov., a novel member of the suborder Micrococcineae from saline soil in China. Int J Syst Evol Microbiol. 54:525–531.

Li WJ, Chen HH, Kim CJ, Zhang YQ, Park DJ, Lee JC, Jiang CL. 2005. *Nesterenkonia sandarakina* sp. nov. and *Nesterenkonia lutea* sp. nov., novel actinobacteria, and emended description of the genus Nesterenkonia. Int J Syst Evol Microbiol. 55:463–466.

Li WJ, Zhang YQ, Schumann P, Liu HY, Yu LY, Zhang YQ, Jiang CL. 2008. *Nesterenkonia halophila* sp. nov., a moderately halophilic, alkalitolerant actinobacterium isolated from a saline soil. Int J. Syst Evol Microbiol. 58:1359–1363.

Luna-Guido M.L. and L. Dendooven. 2001. Simulating the dynamics of glucose and $NH4^+$ in alkaline saline soils of the former Lake Texcoco with the Detran model. Eur J Soil Sci. 52:269–277.

Luna-Guido ML, Vega-Jarquin C, Franco-Hernández MO, Vásquez-Murrieta S, Trujillo-Tapia N, Ramírez-Fuentes E, Dendooven L. 2002. Actividad Microbiana en Suelos [Microbial activity in soils]. Avance y perspectiva. 21:328–332.

Luo HY, Miao LH, Fang C, Yang PL, Wang YR, Shi PJ, Fan YL. 2008. *Nesterenkonia flava* sp. nov., isolated from paper-mill effluent. Int J Syst Evol Microbiol. 58:1927–1930.

Nel AJM, Tuffin IM, Sewell BT, Cowan DA. 2011. Unique aliphatic amidase from a psychrotrophic and haloalkaliphilic *Nesterenkonia* isolate. Appl Environ Microb. 77:3696–3702.

Oarga A. 2009. Life in extreme environments. Revista de Biologia e ciencias da Terra. 9:1–10.

Onishi H, Kamekura M. 1972. *Micrococcus halobius* sp. nov. Int Syst Bacteriol. 22:233–236.

Oren A. 2002. Molecular ecology of extremely halophilic Archaea and Bacteria. FEMS Microbiol Ecol. 39:1–7.

Oren A. 2008. Microbial life at high salt concentrations: phylogenetic and metabolic diversity. Saline Systems. 4:1–13.

Ramírez N, Serrano JA, Sandoval H. 2006. Extremophile microorganisms. Halophile actinomycetes in Mexico. Revista Redalyc. 37:56–71.

Rivadeneyra MA, Delgado G, Soriano M, Ramos-Cormenzana A, Delgado R. 2000. Precipitation of carbonates by *Nesterenkonia halobia* in liquid media. Chemosphere. 41:617–624.

Ruiz-Romero E, Valenzuela-Encinas C, López-Ramírez MP, de Los Angeles Coutiño-Coutiño M, Marsch R, Dendooven L.

2013. *Natronorubrum texcoconense* sp. nov., a haloalkaliphilic archaeon isolated from soil of the former lake Texcoco (Mexico). Arch Microbiol. 195:145–151.

Shafiei M, Ziaee AA, Amoozegar MA. 2010. Purification and biochemical characterization of a novel SDS and surfactant stable, raw starch digesting, and halophilic α-amylase from a moderately halophilic bacterium, *Nesterenkonia* sp. strain F. Process Biochem. 45:694–699.

Shafiei M, Ziaee AA, Amoozegar MA. 2012. Purification and characterization of a halophilic α-amylase with increased activity in the presence of organic solvents from the moderately halophilic *Nesterenkonia* sp. strain F. Extremophiles. 16:627–635.

Soto-Padilla MY, Valenzuela-Encinas C, Dendooven L, Marsch R, Gortarés-Moroyoqui P, Estrada-Alvarado MI. 2014. Isolation and phylogenic identification of soil haloalkaliphilic strains in the former Texcoco Lake. Int J Environ Health Res. 24:82–90.

Stackebrandt E, Koch C, Gvozdiak O, Schumann P. 1995. Taxonomic dissection of the genus *Micrococcus: Kocuria* gen. nov., *Nesterenkonia* gen. nov., *Kytococcus* gen. nov., *Dermacoccus* gen, nov., and *Micrococcus* Cohn 1872 gen. emended. Int J Syst Bacteriol. 45:682–692.

Thacker U, Parikh R, Shouche Y, Madamwar D. 2006. Hexavalent chromium reduction by Providencia sp. Process Biochem. 41:1332–1337.

Valenzuela-Encinas C, Neria-González I, Alcántara-Hernández RJ, Enríquez-Aragón JA, Estrada-Alvarado MI, Hernández-Rodríguez C, Dendooven L, Marsch R. 2008. Phylogenetic analysis of the archaeal community in an alkaline-saline soil of the former lake Texcoco (Mexico). Extremophiles. 12:247–254.

Yoon JH, Jung SY, Kim W, Nam SW, Oh TK. 2006. *Nesterenkonia jeotgali* sp. nov., isolated from jeotgal, a traditional Korean fermented seafood. Int J Syst Evol Micr. 56:2587–2592.

Polish Journal of Microbiology
2018, Vol. 67, No 3, 383–388
https://doi.org/10.21307/pjm-2018-038

SHORT COMMUNICATION

Gut Microbial Compositions in Four Age Groups of Tibetan Minipigs

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Submitted 13 March 2018, revised 12 April 2018, accepted 10 May 2018

Abstract

In this study, the gut microbiota was characterized in four age strata of Tibetan minipigs. Results indicated that the fecal bacteria of 7-, 28-, 56-, and 180-day-old minipigs did not significantly differ in terms of phylogenetic diversity (i.e., PD whole tree) or the Shannon index (both, $p > 0.05$). Findings of a principal coordinate analysis demonstrated that fecal bacteria of 180-day-old minipigs were discernable from those of the other three age groups. From ages seven to 56 days, the abundance of Bacteroidetes or Firmicutes appeared to vary. Regarding genera, the populations of *Bacteroides* and *Akkermansia* decreased with increasing age.

Key words: Tibetan minipig, gut microbiota, age, 16S rRNA gene

In recent decades, numerous studies have addressed the close relationship between gut microbiota and human health (Strati et al. 2017). Minipigs are particularly attractive animal models for gut microbiota research because they are smaller than domestic pigs and therefore cost less to maintain (Pedersen et al. 2013). The composition of gut microbiota has been determined in Ossabaw, Gottingen, and Yucatan minipigs (Pedersen et al. 2013; Val-Laillet et al. 2017); however, different pig breeds have been found to harbor distinct gut microbial profiles (Diao et al. 2016), so breed-specific characterization of intestinal bacterial is important. The Tibetan minipig is distributed primarily in the Tibetan highlands, which have an approximate mean elevation of 4000 m (Yang et al. 2011). The Tibetan minipig is considered a significant native breed in China, owing to its tolerance to crude feed, strong anti-infectious immunity after operation, and robust cardiovascular health (Wu et al. 2012), However, the gut microbiota of this breed has not been characterized previously. Herein, we analyzed the intestinal bacteria of four age groups (7, 28, 56, and 180 days old) of Tibetan minipig by means of 16S rRNA gene sequencing.

The study protocol was approved by the Animal Care and Ethical Committee, Southern Medical University, China (No. L2015126). Two male and three female Tibetan minipigs were obtained from Songshan Lake Pearl Laboratory Animal Science and Technology Co., Ltd., China. All animals were fed a maize and soybean-based diet (Fan et al. 2015) and were weaned at 50 days of age. Fresh fecal samples were collected from each minipig at 7, 28, 56, and 180 days of age. Fecal total DNA was extracted with a fecal DNA nucleic acid extraction kit (Bioeasy Technology Inc., China), in accordance with the manufacturer's instructions. Bacterial 16S rRNA genes were amplified by polymerase chain reaction (PCR) using barcoded universal V4 primers (He et al. 2013). The primer sequences to amplify the V4 hypervariable regions of 16S rRNA genes were V4F (5'-GAGTGCCAGCMGCCGCGGTAA-3') and V4R252 (5'-TTAGGAGACCCGGACTACHVGGGTWTCTAAT-3'). The PCR products were sequenced on a HiSeq 2000 platform (Illumina, San Diego, CA). Sequencing of 200 base pairs (bp) of the 16S rRNA amplicon was carried out from each end. Allowed mismatches were set at less than 10 bp. The sequences were deposited in the European Nucleotide Archive (ENA) with the accession number PRJEB25515.

To preserve sequence quality, no mismatches were permitted in the primer or barcoded regions. Tags with

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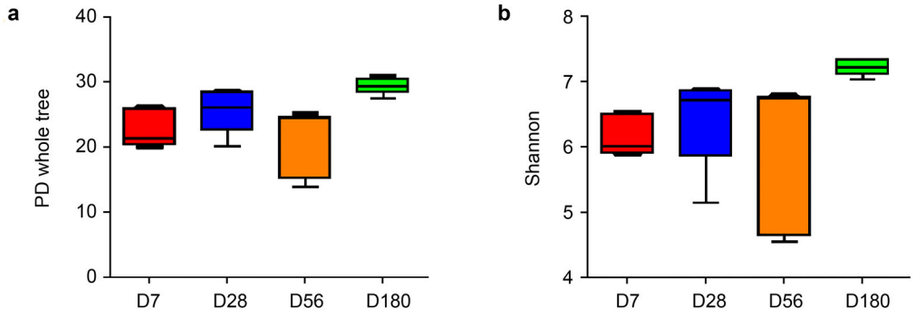

Fig. 1. Alpha diversity comparisons among four age strata. Results of (a) the PD whole tree index and (b) the Shannon index evaluated by Kruskal-Wallis pairwise comparisons.

ambiguous bases were removed (N), and potential chimeric sequences were screened with UCHIME software. Clean, noncontinuous sequences were screened according to BIPES protocol as we have described previously (He et al. 2013). We normalized all samples at the level of 2000 sequences to avoid any uneven

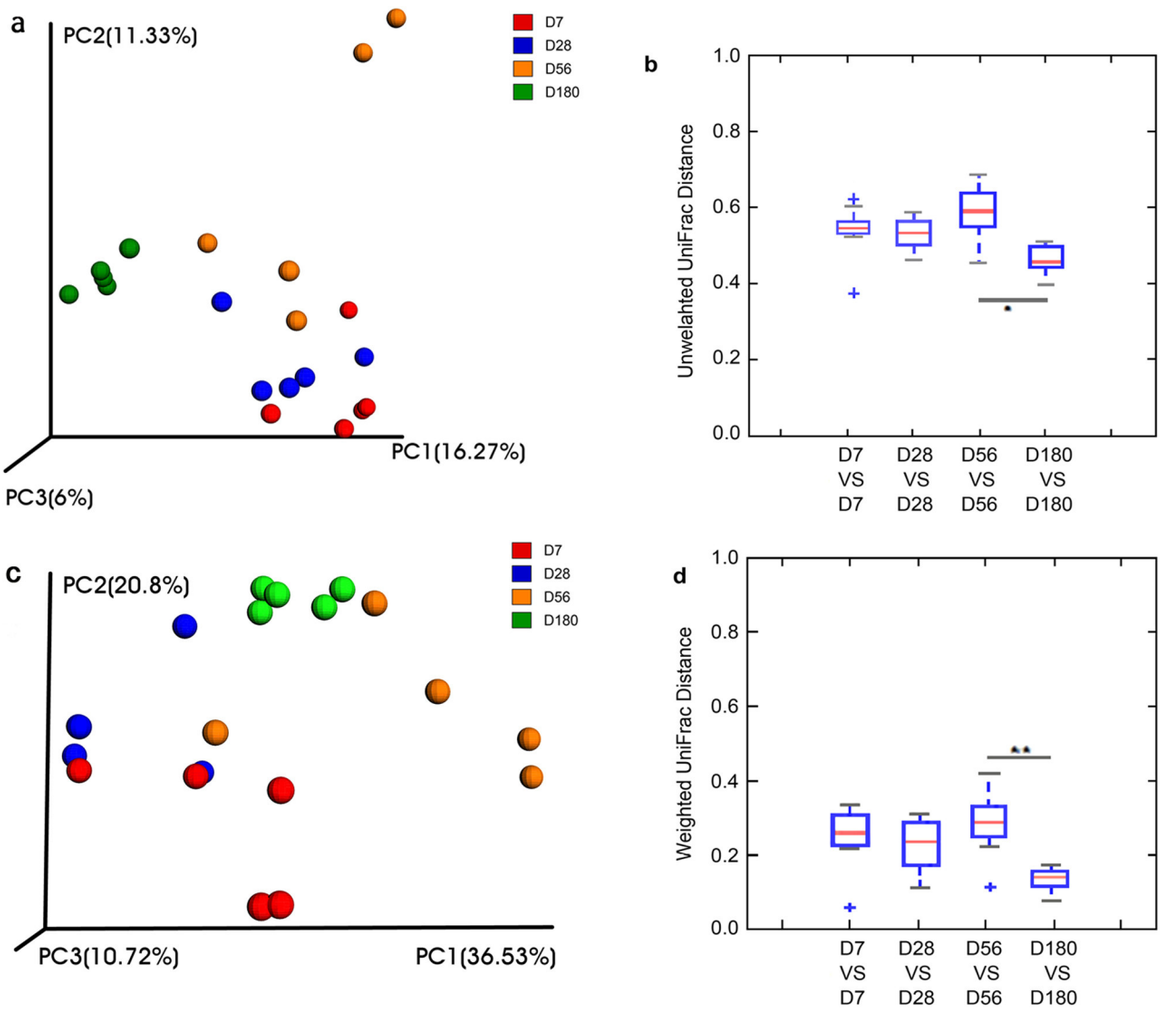

Fig. 2. PCoA of unweighted and weighted UniFrac distances.

(a) PCoA results – calculated with unweighted UniFrac distances – depicting the diversity of gut microbiota among four age groups. (b) Comparisons of unweighted UniFrac distances within each age stratum. (c) PCoA findings – calculated with weighted UniFrac distances – showing the diversity of gut microbiota among 4 age strata. (d) Comparisons of weighted UniFrac distances within each age stratum (*$p<0.05$, ** $p<0.01$, Kruskal-Wallis pairwise comparisons).

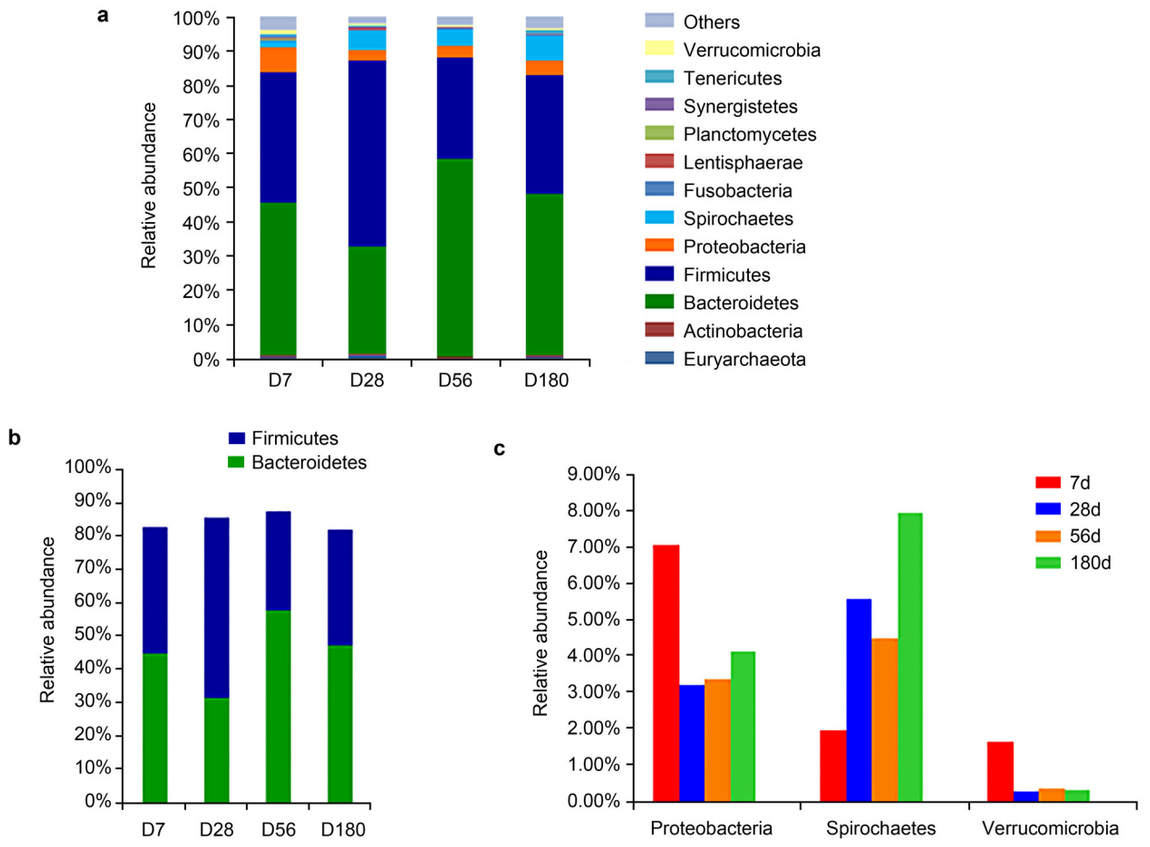

Fig. 3. The average values of relative abundances of phyla within the five animals at each age group (a) phyla. (b) Firmicutes and Bacteroidetes. (c) Proteobacteria, Spirochaetes, and Verrucomicrobia.

sequencing effort between samples. The representative sequence in each operational taxonomic unit (OTU) was assigned a Ribosomal Database Project (RDP) classifier with a similarity threshold of 0.97.

Subsequent analysis was carried out using QIIME version 1.8.0. The PD whole tree and Shannon index were determined as a measure of alpha diversity. Beta diversity was ascertained in terms of the UniFrac distance and the Bray-Curtis dissimilarity distance (He et al. 2013). A principle coordinates analysis (PCoA) based on the UniFrac distance also was performed (Caporaso et al. 2010). Biomarkers of the gut microbiome at specific growth stages were detected by means of linear discriminant analysis effect size (LEfSe) (Segata et al. 2011), a statistical tool used to identify genomic features with complex microbial structure. Statistical analysis was carried out with SPSS version 20.0 (IBM, Armonk, NY). Statistical significance was defined as $p < 0.05$. Prism version 5.0 software (GraphPad, San Diego, CA) was used to prepare graphics.

Results of PD whole tree and Shannon index analyses indicated that the intestinal bacteria of Tibetan minipigs did not differ significantly at ages 7, 28, 56, or 180 days (Fig. 1; both, $p > 0.05$). Beta diversity was obtained by PCoA using weighted or unweighted UniFrac distances (Fig. 2). Our findings indicated that similar trends were obtained with weighted or unweighted UniFrac distances. DNA analysis of fecal samples from 180-day-old minipigs yielded diversity results that differed substantially from those of the other three age groups. Evaluation of bacterial DNA from feces of pigs aged 7, 28, or 56 days gave scattered data – this was especially true for the 56-day-old group. Significant differences in beta diversity were noted when weighted UniFrac distances were applied to comparisons of each 56-day-old minipig with each 180-day-old minipig ($p = 0.009$). Significant differences also were detected when unweighted UniFrac distances were applied to a comparison of each 56-day-old minipig with each 180-day-old minipig ($p = 0.03$). No significant differences were observed for weighted or unweighted UniFrac distances applied to comparisons of the other age groups ($p > 0.05$).

As shown in Fig. 3, among all four age groups, Bacteroidetes and Firmicutes were the predominant phyla in the minipig gut, and the average total abundance of Bacteroidetes and Firmicutes was relatively consistent among the four age groups (82.72%, 85.57%, 87.44%

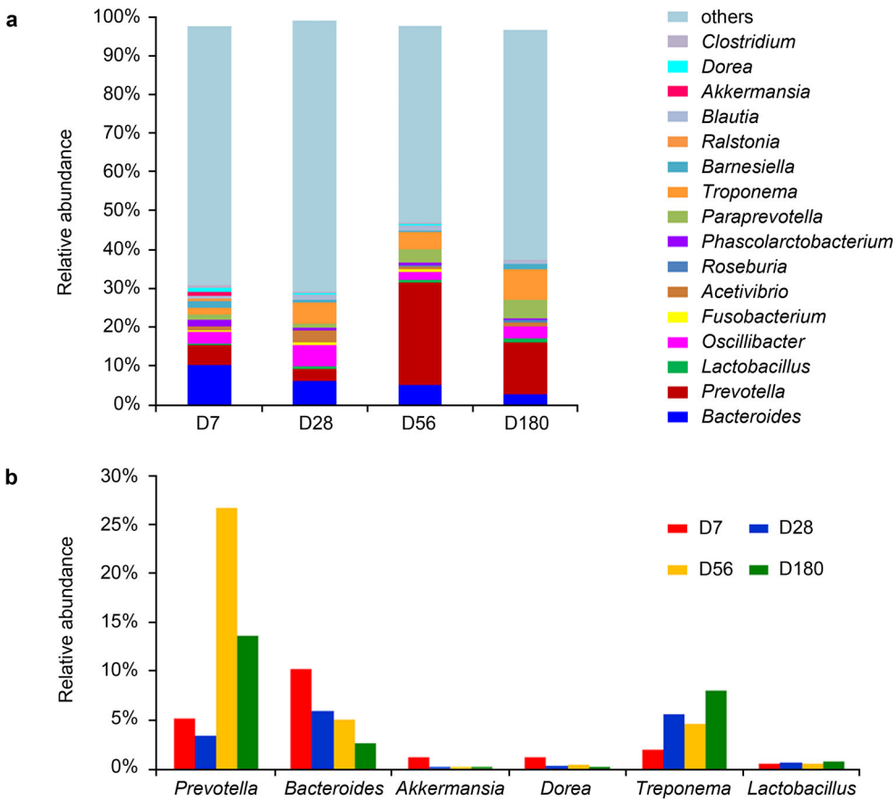

Fig. 4. The average values of relative abundances of genus within the five animals at each age group (a) genus. (b) *Prevotella*, *Bacteroides*, *Akkermansia*, *Dorea*, *Lactobacillus*, and *Treponema*.

and 81.82% respectively). Abundance of Bacteroidetes and Firmicutes varied somewhat in fecal specimens obtained from minipigs aged 7 (44.51% and 38.21%) to 56 days (57.66% and 29.78%). The relative abundances of Proteobacteria and Verrucomicrobia in 7-day-old minipigs exceeded those of the other age groups. The fecal abundance of Spirochaetes increased progressively with age in minipigs from 7 (1.97%) to 180 days (7.96%), with the exception of those aged 56 days (4.52%).

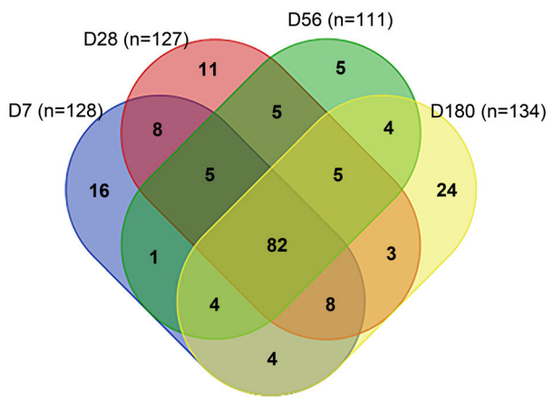

Fig. 5. Venn diagram of genera by age group.

The most dominant bacterial genera populating minipig fecal samples were *Bacteroides*, *Prevotella*, *Oscillibacter*, *Treponema*, *Paraprevotella*, and *Barnesiella*. *Bacteroides* and *Akkermansia* were particularly varied in Tibetan minipigs among the four age groups, and the relative abundances of *Bacteroides* and *Akkermansia* decreased progressively with age (Fig. 4). The relative abundance of *Dorea* was greater in minipigs aged 7 days (1.10%) than in the other age groups (0.10%, 0.35% and 0.15% respectively). The relative fecal abundance of *Prevotella* was greater in minipigs aged 56 days (26.64%) than any other age (5.10%, 3.33% and 13.56% respectively). Compared with the other age categories, minipigs aged 180 days had the greatest relative abundance of *Lactobacillus*. Consistent with the observed trends in Spirochaetes abundance, *Treponema* abundance increased in Tibetan minipigs from 7 days (1.97%) to 180 days (7.96%) of age, with the exception of 56 days (5.57%) of age. As shown in Fig. 5, 82 genera were shared among Tibetan minipigs in the four age strata. Among the age groups, the fewest gut microbial genera were noted in 56-day-old Tibetan minipigs. Findings of microbial marker analyses indicated age-related differences in fecal bacterial of Tibetan minipigs (Fig. 6).

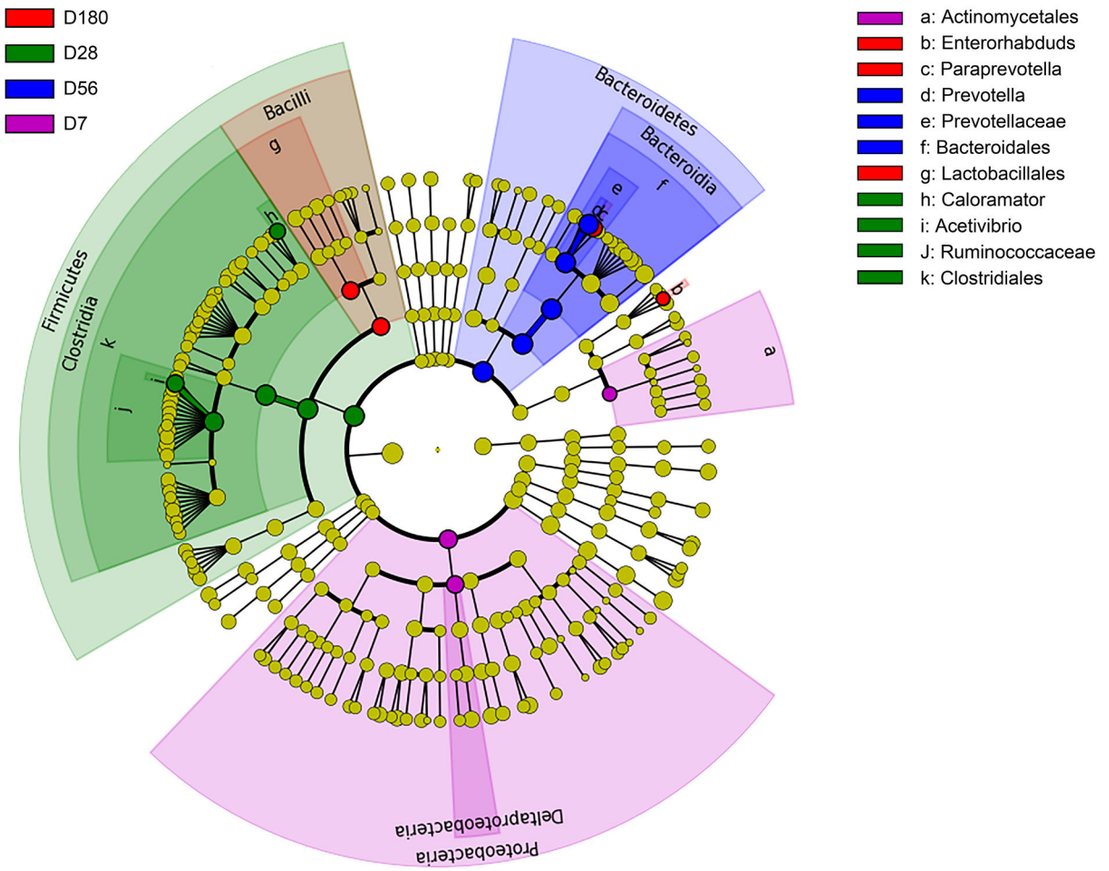

Fig. 6. LEfSe results depicted as a cladogram of bacterial biomarkers in four age strata (linear discriminant analysis [LDA] = 4, $p < 0.05$).

To our knowledge, this study represents the first longitudinal exploration of gut microbial populations of Tibetan minipigs. Unfortunately, a major limitation of the study is that the number of animals is very small, thus the findings and any conclusions require further investigation of more animals before definitive conclusions can be reached.

Results of our alpha diversity analysis indicated that the PD whole tree and Shannon index were highly variable among 56-day-old minipigs. The disparity in alpha diversity among 56-day-old Tibetan minipigs might be attributed to weaning stress, which is likely to affect gut microbial diversity (McLamb et al. 2013). *Prevotella* abundance was found to increase in Tibetan minipigs at 56 days of age, in comparison with the other age strata. The high relative abundance of *Prevotella* in Tibetan minipigs at 56 days of age might be associated with the sudden change in diet resulting from weaning at 50 days of age. After weaning, minipig milk was replaced entirely by a cereal-based diet rich in complex carbohydrates. A diet of solid cereal could affect physiologic conditions of the gut, including the luminal pH and fermentation products. Such changes might be triggered by a rising abundance of *Prevotella* after weaning (Mach et al. 2015).

We noted a higher relative abundance of *Akkermansia* (phylum, Verrucomicrobia) in 7-day-old Tibetan minipigs than in the other age strata. In the intestinal tract of humans and other animals, *Akkermansia* has been found to have protective effects against diseases such as obesity and type I diabetes (Pedersen et al. 2013). Further study is warranted to determine whether *Akkermansia* in Tibetan minipigs has this same function. *Lactobacillus* has been demonstrated to have beneficial effects on the health of humans and other animals; the presence of this genus in the gut is inversely related to obesity and overweight (Pedersen et al. 2013). Herein, we found that *Lactobacillus* abundance was higher in Tibetan minipigs at 180 days of age than in the other age groups. This result was inconsistent with prior clinical findings in which increased age corresponded to decreased *Lactobacillus* abundance (Kumar et al. 2016). Results of our analysis of specific biomarker OTUs suggested that intestinal microorganism abundances are influenced by host age in minipigs. In summary, we report herein the effects of host age on the composition

of gut microbiota in Tibetan minipigs. We advocate further research to evaluate the utility of Tibetan minipigs as a model system for gut microbiota research.

Acknowledgments

This work was supported by the Science and Technology Planning Project of Guangdong Province, China (Grant Nos.: 2010A011200003, 2012B011000004 and 2016A030303008) and the Science and Technology Project of Guangzhou (Grant No.: 201704020012).

Literature

Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Peña AG, Goodrich JK, Gordon JI, et al. 2010. QIIME allows analysis of high-throughput community sequencing data. Nat Methods. 7(5):335–336.

Chen C, Yan LM, Guo KY, Wang YJ, Zou F, Gu WW, Tang H, Li YL, Wu SJ. 2012. The diagnostic value of [18F]-FDG-PET/CT in hematopoietic radiation toxicity: a Tibet minipig model. J Radiat Res (Tokyo). 53(4):537–544.

Diao H, Yan HL, Xiao Y, Yu B, Yu J, He J, Zheng P, Zeng BH, Wei H, Mao XB, et al. 2016. Intestinal microbiota could transfer host gut characteristics from pigs to mice. BMC Microbiol. 16(1):238.

Duranti S, Ferrario C, van Sinderen D, Ventura M, Turroni F. 2017. Obesity and microbiota: an example of an intricate relationship. Genes Nutr. 12(1):18.

Kumar M, Babaei P, Ji B, Nielsen J. 2016. Human gut microbiota and healthy aging: recent developments and future prospective. Nutrition and Healthy Aging. 4(1):3–16.

Lv G, Cheng N, Wang H. 2017. The Gut Microbiota, Tumorigenesis, and Liver Diseases. Engineering. 3(1):110–114.

Mach N, Berri M, Estellé J, Levenez F, Lemonnier G, Denis C, Leplat JJ, Chevaleyre C, Billon Y, Doré J, et al. 2015. Early-life establishment of the swine gut microbiome and impact on host phenotypes. Environ Microbiol Rep. 7(3):554–569.

Matsuoka K, Kanai T. 2015. The gut microbiota and inflammatory bowel disease. Semin Immunopathol. 37(1):47–55.

McLamb BL, Gibson AJ, Overman EL, Stahl C, Moeser AJ. 2013. Early weaning stress in pigs impairs innate mucosal immune responses to enterotoxigenic *E. coli* challenge and exacerbates intestinal injury and clinical disease. PLoS One. 8(4):e59838.

Pedersen R, Ingerslev HC, Sturek M, Alloosh M, Cirera S, Christoffersen BØ, Moesgaard SG, Larsen N, Boye M. 2013. Characterisation of gut microbiota in Ossabaw and Göttingen minipigs as models of obesity and metabolic syndrome. PLoS One. 8(2):e56612.

Segata N, Izard J, Waldron L, Gevers D, Miropolsky L, Garrett WS, Huttenhower C. 2011. Metagenomic biomarker discovery and explanation. Genome Biol. 12(6):R60.

Strati F, Cavalieri D, Albanese D, De Felice C, Donati C, Hayek J, Jousson O, Leoncini S, Renzi D, Calabrò A, et al. 2017. New evidences on the altered gut microbiota in autism spectrum disorders. Microbiome. 5(1):24.

Val-Laillet D, Besson M, Guérin S, Coquery N, Randuineau G, Kanzari A, Quesnel H, Bonhomme N, Bolhuis JE, Kemp B, et al. 2017. A maternal Western diet during gestation and lactation modifies offspring's microbiota activity, blood lipid levels, cognitive responses, and hippocampal neurogenesis in Yucatan pigs. FASEB J. 31(5):2037–2049.

Wu SJ, Wang YJ, Guo KY, Chen C, Zhao TF, Sun MB, Gu WW, Gao YY, Han HJ, Zou F. 2012. Effect of total body X-ray irradiation on lymph node in Tibet minipig. J Radiat Res (Tokyo). 53(1): 18–23.

Yang S, Zhang H, Mao H, Yan D, Lu S, Lian L, Zhao G, Yan Y, Deng W, Shi X, et al. 2011. The local origin of the Tibetan pig and additional insights into the origin of Asian pigs. PLoS One. 6(12):e28215.

Zeng G, Liu R, Liao H, Zhang X, Qian Y, Liu B, Wu Q, Zhao J, Gu W, Li H. 2013. Single Intraperitoneal Injection of Monocrotaline as a Novel Large Animal Model of Chronic Pulmonary Hypertension in Tibet Minipigs. PLoS One. 8(11):e78965.

Polish Journal of Microbiology
2018, Vol. 67, No 3, 389–395

POLSKIE TOWARZYSTWO MIKROBIOLOGÓW

INFORMACJE Z POLSKIEGO TOWARZYSTWA MIKROBIOLOGÓW

Poniżej w punktach, w formie skrótowej przedstawiono omawiane sprawy na wirtualnych zebraniach Prezydium ZG PTM oraz spotkaniach z udziałem członków Prezydium.

1. PTM otrzymało dofinansowanie z MNiSW na Działalność Upowszechniającą Naukę związaną z modernizacją w latach 2018–2019 czasopism PTM: *Polish Journal of Microbiology* i *Postępów Mikrobiologii* na kwotę 65 200 zł. Otrzymane środki będą przeznaczone zgodnie z zadaniami określonymi w zaakceptowanym przez MNiSW wniosku na:
   a) zakup puli numerów DOI dla publikowanych artykułów *PJM*
   b) zakup puli numerów DOI dla publikowanych artykułów *PM*
   c) wdrożenie nowoczesnej platformy publikacji czasopisma *PJM*
   d) profesjonalne tłumaczenie artykułów z języka polskiego na angielski dla *PM*
2. Podpisano umowę z firmą z Lublina na tłumaczenie na język angielski prawie 500 stron maszynopisów prac składanych do *Postępów Mikrobiologii* do zeszytów 3 i 4 w 2018 r.
3. Złożono wniosek do Biblioteki Narodowej i otrzymano numer ISSN dla wydawanej tylko on-line wersji *Postępy Mikrobiologii*. Docelowo chcemy, aby wersja on-line w dużej mierze zawierała publikacje w języku angielskim. Powinna wtedy poprawić się cytowalność publikacji kwartalnika oraz wzrosnąć jego Impact Factor.
4. W toku są prace związane z tworzeniem anglojęzycznej strony czasopisma *Postępy Mikrobiologii*.
5. Przekazano pełnomocnictwo radcom prawnym do reprezentowania ZG PTM w obszarze uaktualnienia danych w KRS dotyczących czasopism PTM – *Polish Journal of Microbiology* oraz *Postępy Mikrobiologii*.
6. Zakończyły się działania związane z wprowadzeniem nowego Statutu PTM. Został on wpisany do Krajowego Rejestru Sądowego. Nowy Statut PTM z dnia 23.09.2017 r. jest dostępny na stronie internetowej PTM.
7. W oparciu o nowy Statut PTM z dnia 23.09.2017 r. została uporządkowana lista członków PTM. W dniu 21.06.2018 r. wysłaliśmy zawiadomienie o zamiarze skreślenia z listy członków PTM (drogą listowną – 22 osoby, drogą elektroniczną – 73 osoby) do członków zalegających z opłatą za składki członkowskie. Ostatecznie Uchwałą nr 29–2018 zostało ponad 80 osób usuniętych z grona członków PTM za niepłacenie składek członkowskich.
8. Dnia 16 lipca 2018 r. odbyło się internetowe głosowanie 5 Uchwał Prezydium ZG PTM. Uchwałą nr 28–2018 przyjęto 12 nowych członków zwyczajnych PTM.
9. W dniach 7–8 września w Warszawie odbyło się spotkanie „45 FEMS" zorganizowane po raz pierwszy w naszym kraju przez Polskie Towarzystwo Mikrobiologów. Na nasze zaproszenie przyjechali przedstawiciele 35 europejskich towarzystw mikrobiologicznych reprezentujących: Armeńskie Towarzystwo Mikrobiologiczne; Stowarzyszenie na rzecz Mikrobiologii Ogólnej i Stosowanej; Austriackie Towarzystwo Molekularnych Nauk Przyrodniczych i Biotechnologii; Białoruskie Pozarządowe Towarzystwo Mikrobiologów; Belgijskie Towarzystwo Mikrobiologiczne, Brytyjskie Towarzystwo Mykologiczne; Brytyjskie Towarzystwo Fykologiczne; Bułgarskie Towarzystwo Mikrobiologiczne (Unia Naukowców w Bułgarii); Chorwackie Towarzystwo Mikrobiologiczne; Duńskie Towarzystwo Mikrobiologiczne; Estońskie Towarzystwo Mikrobiologiczne; Fińskie Towarzystwo Biochemiczne, Biofizyczne i Mikrobiologiczne; Francuskie Towarzystwo Mikrobiologiczne; Niemieckie Towarzystwo Higieny i Mikrobiologii; Międzynarodowe Stowarzyszenie Biodeterioracji i Biodegradacji; Międzyregionalne Rosyjskie Towarzystwo Mikrobiologiczne; Włoskie Towarzystwo Mikrobiologii Klinicznej; Włoskie Stowarzyszenie Przemysłu Rolno-Spożywczego i Mikrobiologii Środowiskowej; Włoskie Towarzystwo Mikrobiologii Ogólnej i Biotechnologii Mikrobiologicznej; Łotewskie Towarzystwo Mikrobiologiczne; Litewskie Towarzystwo Mikrobiologiczne; Towarzystwo Mikrobiologiczne (dawniej Society for

General Microbiology); Norweskie Towarzystwo Mikrobiologiczne; Portugalskie Towarzystwo Mikrobiologiczne; Holenderskie Królewskie Towarzystwo Mikrobiologiczne; Serbskie Towarzystwo Mikrobiologiczne; Słoweńskie Towarzystwo Mikrobiologiczne; Towarzystwo Mikrobiologii Stosowanej; Towarzystwo Wirusologiczne (z Niemiec); Towarzystwo Mikrobiologów Ukrainy; Towarzystwo Mikrobiokosmosu; Hiszpańskie Towarzystwo Mikrobiologiczne; Szwajcarskie Towarzystwo Mikrobiologiczne; Tureckie Towarzystwo Mikrobiologiczne.

Na spotkanie przyjechał również cały zarząd FEMS – Prezydent FEMS: prof. Bauke Oudega z Holandii oraz 7 dyrektorów FEMS wraz z 12 pracownikami FEMS.

Obrady odbywały się w hotelu Sofitel Victoria w Warszawie i wspierane były przez dwójkę wolontariuszy – członków PTM, dr Annę Pietruczuk-Padzik i mgr Karolinę Stępień z Warszawskiego Uniwersytetu Medycznego. Logistycznie FEMS Council przygotowywany był i nadzorowany przez firmę Global Congress Sp. z o.o., a w trakcie obrad pomoc zapewniały 2 Panie z tej firmy. Uczestnicy FEMS Council powitani zostali na „Welcome reception" i pożegnani kolacją w restauracji AleGloria na Placu Trzech Krzyży. W wolnym czasie uczestnicy mieli zorganizowany spacer po Starówce Warszawy z przewodnikami.

Koszty obrad „45 FEMS Council" pokrywane były przez zarząd FEMS, natomiast imprezy towarzyszące finansowane były w ½ przez zarząd FEMS, natomiast ¼ przez PTM i ¼ przez firmę Global Congress Sp. z o.o. Materiały promujące Warszawę i 3 przewodników udostępniła nieodpłatnie firma Warsaw Convention Bureau z Urzędu m. st. Warszawy, za co bardzo dziękujemy.

Otrzymałem szereg e-maili z podziękowaniami za bardzo udane spotkanie w Warszawie, za świetną organizację, dobre hotele i jedzenie, a także za możliwość zobaczenia Warszawy. Wielu uczestników FEMS Council było po raz pierwszy nie tylko w Warszawie, ale także w Polsce.

10. W połowie września został złożony do MNiSW wniosek na Działalność Upowszechniającą Naukę związaną z planowaną konferencją: MAKRO-kierunki w MIKRO-biologii – Konferencja z okazji 70-lecia Oddziału Warszawskiego Polskiego Towarzystwa Mikrobiologów. Konferencja odbywać się będzie 27 września 2019 r. na terenie Wydziału Biologii Uniwersytetu Warszawskiego. Przewodniczącym Komitetu Organizacyjnego jest Pan dr Tomasz Jagielski Przewodniczący Oddziału Warszawskiego PTM.
11. Przypominamy, uchwałę Prezydium ZG PTM z dnia 30.08.2017:
„Wprowadza się możliwość ponownego przystąpienia do PTM osobom skreślonym z listy członków zwyczajnych z powodu niepłacenia składek członkowskich, po rocznym okresie karencji, od 01.10.2018 r."
Uchwała dotyczy osób, które z powodu długoletnich zaległości w opłacaniu składek zostały usunięte z grona członków PTM po Nadzwyczajnym Walnym Zebraniu Delegatów PTM, które odbyło się we wrześniu ub. r. w Krakowie.
12. Osoby, które zostały skreślone po 1.10.2017 r. mogą przystąpić ponownie do PTM zgodnie ze Statutem PTM z dnia 23.09.2016 r., paragraf 16.
13. Prezydium ZG PTM wraz z Komisją Konkursową przygotowało materiały (w tym Regulamin Konkursu) związane z konkursem o Nagrodę Naukową PTM im. prof. Edmunda. Mikulaszka. W Uchwale nr 32–2018 Prezydium ZG PTM podjęło decyzję w sprawie przyjęcia Regulaminu i materiałów towarzyszących, dotyczących Nagrody Naukowej PTM im. Prof. Edmunda Mikulaszka.

Nagroda w 2018 r. dotyczy członków PTM, autorów publikacji, które ukazały się drukiem w latach 2016–2017. Wszystkie informacje o konkursie znajdują się poniżej oraz na stronie internetowej PTM.

Termin składania wniosków: 31.10.2018 r.
Ogłoszenie wyników konkursu: 15.12.2018 r.

MATERIAŁY ZWIĄZANE Z KONKURSEM O NAGRODĘ NAUKOWĄ PTM IM. PROF. EDMUNDA MIKULASZKA

Nagroda Naukowa Polskiego Towarzystwa Mikrobiologów im. prof. Edmunda Mikulaszka

Pan Profesor Edmund Mikulaszek był wybitnym mikrobiologiem polskim. Urodził się 21 września 1895 roku we Lwowie. Studiował medycynę na Wydziale Lekarskim Uniwersytetu Jana Kazimierza we Lwowie, gdzie uzyskał dyplom doktora wszech nauk lekarskich, a następnie stopień naukowy docenta mikrobiologii i serologii. Był m.in. kierownikiem Katedry Mikrobiologii Lwowskiego Instytutu Weterynaryjnego, filii Państwowego Zakładu Higieny we Lwowie i Sanitarno-Epidemiologicznego Laboratorium Frontu. Po wojnie zorganizował i objął kierownictwo Zakładu Mikrobiologii Wydziału Lekarskiego Uniwersytetu Warszawskiego, a następnie Akademii Medycznej w Warszawie. Przewodniczył m.in. Komitetowi Mikrobiologii Polskiej Akademii Nauk, Radzie Naukowej Wojskowego Instytutu Higieny i Epidemiologii oraz podkomisji surowic i szczepionek Komisji Farmakopei Polskiej. Ogłosił ok. 200 prac z dziedziny mikrobiologii, immunologii i immunochemii, większość w czasopismach zagranicznych. Wielokrotnie reprezentował naukę polską na kongresach i konferencjach międzynarodowych, wyszkolił wielu samodzielnych pracowników naukowych. W okresie międzywojennym za udział w wojnie w 1920 roku został odznaczony medalem i Złotym Krzyżem Zasługi, za udział w II wojnie – Krzyżem Grunwaldu III klasy, po wojnie – Krzyżem Oficerskim i Komandorskim z Gwiazdą Orderu Odrodzenia Polski. Nad wyraz skromny, prawy, sumienny, życzliwy ludziom, o dużej kulturze osobistej, bez reszty oddany nauce, darzony powszechnym szacunkiem środowiska. Zmarł po ciężkiej chorobie 26 sierpnia 1978 roku, w wieku 83 lat, pochowany został z asystą wojskową na Powązkach (*Med Dośw Mikrobiol* 1993, 45, 529–531).

Przez spadkobierców Profesora została ufundowana Nagroda Jego Imienia przyznawana przez Polskie Towarzystwo Mikrobiologów.

Nagroda jest przyznawana co dwa lata za prace doświadczalne z zakresu szeroko pojętej mikrobiologii, wykonane przez młodych pracowników nauki, członków PTM, którzy w chwili wnioskowania o nagrodę nie przekroczyli 35 roku życia i nie byli w tym okresie samodzielnymi pracownikami naukowymi. Współautorami prac mogą być profesorowie lub doktorzy habilitowani bez prawa wyróżnienia nagrodą pieniężną, natomiast z prawem do wyróżnienia okolicznościowym dyplomem. Prace powinny być wykonane w pracowniach na terenie kraju. Współautorami prac przedstawionych do Nagrody nie mogą być autorzy zagraniczni.

Prace do Nagrody są zgłaszane przez autora bezpośrednio zainteresowanego.

Przewidziane są nagrody pieniężne w wysokości:

I stopnia – 5.000 zł

II stopnia – 3.000 zł

III stopnia – 2.000 zł

Komisja konkursowa składa się z 7 osób: przewodniczącego, 2 redaktorów naczelnych czasopism PTM – *Postępy Mikrobiologii* i *Polish Journal of Microbiology* oraz 4 członków.

Nagrody im. prof. Mikulaszka będą wręczane na najbliższym Zjeździe PTM, a Laureat Nagrody zobowiązany jest do prezentacji nagrodzonej pracy na tym Zjeździe. Zarząd Główny PTM może podjąć decyzję o innej formie wręczenia nagrody.

Szczegółowe informacje dotyczące zasad Konkursu przedstawia Regulamin Nagrody Naukowej PTM im. prof. Edmunda Mikulaszka zamieszczony na stronie internetowej PTM: www.microbiology.pl

Skład Komisji w edycji Konkursu w 2018 r.:

- prof. dr hab. Stefania Giedrys-Kalemba – Przewodnicząca Komisji
- prof. dr hab. Elżbieta Anna Trafny – Redaktor Naczelny P*olish Journal of Microbiology*
- prof. dr hab. Jacek Bielecki – Redaktor Naczelny *Postępy Mikrobiologii*
- prof. dr hab. Małgorzata Bulanda – Członek Komisji
- prof. dr hab. Wiesław Kaca – Członek Komisji
- dr hab. prof. nadzw. UŁ Beata Anna Sadowska – Członek Komisji
- dr hab. prof. nadzw. PG Beata Krawczyk– Członek Komisji

REGULAMIN
Nagrody Naukowej im. prof. Edmunda Mikulaszka ustanowionej przez Polskie Towarzystwo Mikrobiologów

1. Nagroda jest przyznawana, co dwa lata za prace doświadczalne z zakresu szeroko pojętej mikrobiologii, wykonane przez młodych pracowników nauki, będących w roku ukazania się publikacji oraz aktualnie członkami PTM, którzy w chwili wnioskowania o nagrodę nie przekroczyli 35 roku życia i nie byli w tym okresie samodzielnymi pracownikami naukowymi.
2. Nagrodę przyznaje się za pracę doświadczalną lub cykl prac doświadczalnych ściśle ze sobą tematycznie powiązanych, opublikowanych w okresie dwóch lat poprzedzających rok przyznawania nagród.
3. Prace powinny być wykonane w pracowniach na terenie kraju i mieć afiliację polskiej instytucji naukowej, współautorami nie mogą być autorzy zagraniczni.
4. Autor wnioskujący o nagrodę (wzór – Zał. 1) powinien mieć decydujący wkład w wykonanie i przygotowanie prac:
   a) w przypadku kiedy jest jeden wnioskodawca – przy 2 współautorach co najmniej 60%, przy trzech i więcej współautorach, co najmniej 40%. Ponadto powinien być pierwszym autorem – w pojedynczej publikacji zgłaszanej do nagrody lub w większości publikacji zgłaszanego cyklu (wzór – Zał. 2).
   b) w przypadku kiedy jest dwóch wnioskodawców – kiedy są dwoma współautorami tej samej publikacji, a ich wkład wynosi po 50%; kiedy jest 3 autorów to wkład każdego z wnioskodawców wynosi co najmniej 40%. Ponadto w przypadku pracy z 3 autorami jeden z wnioskodawców powinien być pierwszym autorem (wzór – Zał. 2).
5. Współautorami prac mogą być profesorowie lub doktorzy habilitowani bez prawa wyróżnienia nagrodą pieniężną, natomiast z prawem do wyróżnienia okolicznościowym dyplomem. Wkład autora wnioskującego winien być potwierdzony oświadczeniami współautorów (wzór – Zał. 2).
6. Zarząd Główny PTM podejmuje Uchwałę o kolejnej edycji Konkursu o Nagrodę Naukową im. prof. Edmunda Mikulaszka i ogłasza na stronie internetowej PTM miejsce oraz termin zgłaszania prac do Konkursu.
7. Prace do Nagrody są zgłaszane przez autora bezpośrednio zainteresowanego drogą e-mailową do Przewodniczącej Komisji Konkursowej Pani Prof. dr hab. Stefanii Giedrys-Kalemba – e-mail: kalemba@mp.pl oraz do biura PTM – e-mail: ptm.zmf@wum.edu.pl
8. Warunkiem przyjęcia pracy/cyklu prac do Nagrody jest złożenie:
   a) wniosku zawierającego dane dotyczące autora zgłoszonego do nagrody pieniężnej lub autorów (wzór – Zał. 1),
   b) odbitek publikacji,
   c) oświadczenia współautorów o wyrażeniu zgody na złożenie wniosku (wzór – Zał. 2).
9. Postępowanie kwalifikacyjne będzie przeprowadzone w ciągu 6 tygodni od upływu terminu zgłaszania prac. Etapy postępowania Komisji Konkursowej:
   – Przewodniczący Komisji Konkursowej dokonuje wstępnej oceny wniosków, sprawdza w biurze PTM okres przynależności kandydata do PTM, następnie wnioski spełniające warunki formalne przekazuje drogą e-mailową pozostałym członkom komisji celem wytypowania najlepszych prac.
   – Każdy z członków Komisji ustala według własnej oceny ranking nadesłanych wniosków. Ocena wniosków jest dokonywana na podstawie regulaminu pracy komisji oceniającej. Suma przyznanych przez wszystkich oceniających punktów decyduje ostatecznie o rankingu wniosków. W przypadku uzyskania jednakowej liczby punktów decyzję o przyznaniu Nagrody podejmuje przewodniczący Komisji Konkursowej.
   – Członek Komisji Konkursowej nie uczestniczy w ocenie pracy, w której on sam lub jego bezpośredni podwładni są współautorami. W takim przypadku sumę punktów przyznanych wnioskowi przez pozostałych członków Komisji powiększa się o średnią tych ocen.
10. Przewidziane są nagrody pieniężne w wysokości:
    I stopnia – 5.000 zł
    II stopnia – 3.000 zł
    III stopnia – 2.000 zł
    W przypadku podania kilku osób do nagrody, gratyfikacja finansowa będzie dzielona równo pomiędzy współautorów.
11. Ustalenia Komisji Konkursowej przekazywane są do Zarządu Głównego PTM, który podejmuje stosowną Uchwałę.
12. Nagrody Naukowe PTM im. prof. E. Mikulaszka są wręczane na najbliższym Zjeździe PTM, a Laureat Nagrody zobowiązany jest do prezentacji nagrodzonej pracy/cyklu prac na tym zjeździe. Zarząd Główny PTM może podjąć decyzję o innej formie wręczenia Nagrody.

13. Złożenie Wniosku o Nagrodę Naukową PTM im. prof. Edmunda Mikulaszka jest równoznaczne z wyrażeniem zgody na przetwarzanie danych w celu udziału w Konkursie o Nagrodę Naukową im. prof. Edmunda Mikulaszka.

Regulamin zatwierdzono Uchwałą nr 32–2018 Prezydium Zarządu Głównego PTM dnia 16.07.2018 r.

Załącznik 1
do REGULAMINU Nagrody Naukowej im. prof. Edmunda Mikulaszka
ustanowionej przez Polskie Towarzystwo Mikrobiologów

Wniosek
o Nagrodę Naukową PTM im. prof. Edmunda Mikulaszka

Dane autora wnioskującego o nagrodę pieniężną [*w przypadku kilku autorów (np. 2) należy wypełnić kilka wniosków (2) i załączyć kilka (2) oświadczeń osobno dla każdego z wnioskujących współautorów*].

Imię i nazwisko autora/współautora*: .....
Data urodzenia:. .....
Tytuł zawodowy, stopień naukowy: .....
Nazwa i adres placówki, w której pracę wykonano: .....
Nazwa i adres instytucji zatrudniającej autora publikacji: .....
Adres zamieszkania, telefon kontaktowy, e-mail: .....
Zgłaszana praca/wykaz prac (*IF, MNiSW w roku opublikowania*): .....
Uzasadnienie wniosku: (*od 50 do 200 słów*) .....

Uwagi:
* niepotrzebne skreslić

Oświadczam, że w roku opublikowania pracy/prac byłem członkiem PTM i jestem nim obecnie.

Data ....
Podpis

Obowiązek infromacyjny

Administratorem Pani/Pana danych osobowych podanych w związku z ubieganiem się o Nagrodę Naukową PTM im. prof. Edmunda Mikulaszka jest Polskie Towarzystwo Mikrobiologów z siedzibą w Warszawie, adres: ul. Stefana Banacha 1B, 02-097, Warszawa, KRS 0000006649 (dalej: PTM). Pani/Pana dane osobowe będą przetwarzane na podstawie: art. 6 ust. 1 lit. a Rozporządzenia Parlamentu Europejskiego i Rady (UE) 2016/679 z dnia 27 kwietnia 2016 r.; (RODO) tj. Pani/Pana zgody na przetwarzanie danych osobowych, pod którą rozumiane jest złożenie ww. wniosku jak również na podstawie art. 6 ust. 1 lit f RODO, tj. jest to niezbędne do celów wynikających z prawnie uzasadnionych interesów realizowanych przez administratora jakim jest obrona przed ewentualnymi roszczeniami.

Pani/Pana dane osobowe będą przetwarzane w celu rozpatrzenia wniosku i udziale w Konkursie o Nagrodę Naukową im. prof. Edmunda Mikulaszka. Podanie danych jest dobrowolne lecz ich niepodnie uniemożliwi udział w konkursie. Pani/Pana dane będą udostępniane uprawnionym na podstawie przepisów prawa organom państwowym. Pani/Pana dane będą również przekazane podmiotom przetwarzającym takim jak podmioty świadczące na naszą rzecz usługi IT.

Pani/Pana dane osobowe będą przetwarzane do dnia rozstrzygnięcia Konkursu o Nagrodę Naukową im. prof. Edmunda Mikulaszka, a następnie przez okres niezbędny do ustalenia, dochodzenia lub obrony roszczeń (tj. przez czas przedawnienia tych roszczeń). Przysługuje Pani/Panu prawo dostępu do swoich danych osobowych, żądania ich sprostowania lub usunięcia. Ponadto przysługuje Pani/Panu prawo do żądania ograniczenia przetwarzania, prawo sprzeciwu wobec ich przetwarzania oraz prawo do przenoszenia danych. W każdym momencie może Pani/Pan wycofać wyrażoną zgodę, w takim wypadku udział w konkursie będzie jednak niemożliwy. Przysługuje Pani/Panu również prawo wniesienia skargi do Prezesa Urzędu Ochrony Danych Osobowych.

Załącznik 2
do REGULAMINU Nagrody Naukowej im. prof. Edmunda Mikulaszka
ustanowionej przez Polskie Towarzystwo Mikrobiologów

Oświadczenie

Oświadczam, że autor .....
miał decydujący wkład (Regulamin Nagrody) zgodnie z opisem indywidualnego wkładu ww. autora w jej/ich współautorstwo w wykonanie i przygotowanie niżej wymienionej pracy/prac:

1. (*Autorzy, tytuł artykułu, nazwa czasopisma, rok, tom, strony.*
   Opis indywidualnego wkładu oraz oszacowany % udziału w danej publikacji wnioskującego o nagrodę)

2. (*Autorzy, tytuł artykułu, nazwa czasopisma, rok, tom, strony.*
   Opis indywidualnego wkładu oraz oszacowany % udziału w danej publikacji wnioskującego o nagrodę)

3. (*Autorzy, tytuł artykułu. nazwa czasopisma. rok, tom, strony.*
   Opis indywidualnego wkładu oraz oszacowany % udziału w danej publikacji wnioskującego o nagrodę)

   i wyrażam zgodę na zgłoszenie jej/ich do Nagrody PTM im. prof. Edmunda Mikulaszka.

Data ....
Podpis

Warszawa 18.09.2018 r.

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

PREZES
Polskiego Towarzystwa Mikrobiologów
prof. dr hab. Stefan Tyski

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T: +31 (0) 15 302 0050 E: fems@fems-microbiology.org W: www.fems-microbiology.org

20 September 2018

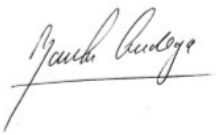
Dear Stefan,

RE: FEMS Council 2018, Warsaw

I would like to send formal thanks on behalf of FEMS for all of your efforts in organising the 2018 Council meeting. It was a great success as a consequence of your enthusiasm and hard work. The organization, venue, and accommodation, were flawless and contributed to an event that was as enjoyable as it was useful and productive. I received many positive comments about the meeting and the location from attendees. I hope that you enjoyed the event as much as we did. I particularly enjoyed the social programme, enabling me to see much of Warsaw on my first visit to the city. I would also like to pass on my thanks to the volunteers who contributed to the smooth running of everything.

I appreciated your presentation on the history and background of Poland and the Polish Society of Microbiologists, and it seems particularly poignant to have the Council meeting in Warsaw during the centenary of an independent Poland. Warsaw is a wonderful city and I look forward to returning one day.

Warm regards



Bauke Oudega

President, Federation of European Microbiological Societies

cc Catherine Cotton, CEO

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