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Post-Conference Communications and Articles

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Editorial
VI PhD Students National Conference of Life Sciences "BioOpen"

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The PhD Students National Conference of Life Sciences – "BioOpen" was held for the first time at the Faculty of Biology and Environmental Protection of the University of Lodz in 2015. The conference was organized by doctoral students of the Faculty of Biology and Environmental Protection, University of Lodz, supported by the faculty staff. The patronage over this event was taken by: Rector of the University of Lodz, Prof. Włodzimierz Nykiel, Dean of the Faculty of Biology and Environmental Protection, Prof. Elżbieta Żądzińska, Polish Biophysical Society, as well as the Director of the Institute of Medical Biology of the Polish Academy of Sciences, Prof. Jarosław Dziadek. This event was also part of the Doctoral Science Week initiative and the 15th Festival of Science, Technology and Art. During the conference, lectures were presented by outstanding guests from the world of science and young scientists from all over the country. The conference was a place for presenting the achievements of numerous research teams, exchanging valuable experiences in the field of research and demonstrating the achievements of young doctoral students. During these conference, doctoral students inspired each other to seek answers to their questions.

Due to the COVID-19 situation, after a one-year break, it was decided to organize this year's edition of the conference on-line, without having to pay participation fees. The conference was divided into four thematic sessions: ecology and environmental protection, biotechnology and genetic engineering, molecular and medical biology as well as microbiology and immunology.

The wide thematic profile of the conference allowed to show the diversity of research work in the field of biology, which combines works in the field of microbiology, biotechnology, biochemistry, biophysics, plant and animal physiology, cell biology and other disciplines. The possibility of linking biological work with other disciplines was also shown, which may result in the creation of interdisciplinary projects in the future.

Folia Biologica et Oecologica is a journal dedicated mainly to young scientists, to whom we can offer support during the entire publication process, as well as professional linguistic proofreading. Therefore, already in 2019, talks were initiated with the organizers of the conference for young scientists that Folia Biologica et

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Oecologica will participate in the "BioOpen" conference organized at the Faculty of Biology and Environmental Protection of the University of Lodz.

Thanks to the obtained funding from the Ministry of Science and Higher Education, as part of the "Support for scientific journals" program, we have provided free, professional proofreading in English of each scientific article and post-conference application accepted for publication. We hope that in this way we will help many young scientists to start their scientific careers and encourage them to continue publishing in English in journals with an international range.

This special issue includes both review and research articles on the latest advances and fundamental principles in the broadly understood field of biology. The guest editors are very grateful to those who have made their contribution to this edition with manuscripts, as well as to those who have expertly reviewed it. We hope that the next benefit of this issue will be to arouse the interest of the scientific community composed of advanced researchers and academic students in the topics of young researchers publications.

Folia Biologica et Oecologica 17: 7–8 (2021)

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The development of multidrug resistance in cancer cells: the potential of ABC transporter-targeted therapy to overcome inefficiency of treatment

BIOOPEN 2021 – POST-CONFERENCE COMMUNICATION

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Conventional chemotherapy has been widely used as a first-line treatment for cancer patients. On the one hand, these drugs are efficient against all types of cancer because of their non-selective biodistribution; on the other hand, their usage is limited by side effects (Janmaat *et al*. 2017; Kim and Khang 2020). Chemoresistance, as a result of increasing DNA repair or overexpression of ATP-binding cassette (ABC) transporters, constitutes another obstacle (Kim and Khang 2020).

ABC proteins constitute a highly conserved and ubiquitous family of proteins. Forty-eight genes and one pseudogene encoding these proteins have been classified into seven subfamilies (A–G) according to their sequence and structure similarity (Gomez-Zepeda *et al*. 2020). ABC transporters utilize energy derived from ATP hydrolysis to transport molecules across the plasma membrane against their gradient. Expression of ABC transporters has been detected in sundry tissues, especially relevant to biological barriers; these proteins are responsible for absorption, distribution and elimination of the drug (Adamska and Falasca 2018; Bloise *et al*. 2016). The overexpression of genes encoding ABC transporters has been observed in many types of cancer and is related to the presence of multidrug resistance (MDR) (Adamska and Falasca 2018; Fultang *et al*. 2020).

MDR is the process of resistance to a broad spectrum of structurally diverse compounds and is a major cause for the inefficiency of chemotherapy (Adamska and Falasca 2018). ABC transporters, as potent efflux pumps, remove drugs from cancer cells and thereby reduce the drug's effect. Therefore, accumulation of anticancer substrate is limited by ABC transporter activity (Dantzic *et al*. 2018; Goldstein 1995). Studies on a bat-derived cell line have shown that knockdown of the ABCB1 gene or culture with verapamil, an ABCB1 inhibitor, significantly decreases cell viability. These results suggest that higher expression of the ABCB1 gene is connected with excessive drug efflux (Koh *et al*. 2019); ABCB1 overexpression makes treatment failure three times more likely (Choi and Yu 2014; Trock *et al*. 1997). Overexpression of ABC transporters has also been reported in cancer stem cells (CSCs) that occur in cancers with enhanced tumorigenic potential and MDR (Begicevic and Falasca 2017).

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Therefore, exploring the genes encoding ABC proteins could contribute to solving the problems of chemotherapy failure (Kim and Khang 2020).

Inhibitors of ABC transporters, such as PSC-833, GF120918, verapamil or tyrosine kinase inhibitors, might modulate the activity of these proteins and promote the intracellular accumulation of drugs (Wu and Fu 2018). At the same time, there is no successful and safe agent to counteract MDR via inhibiting the activity of ABC transporters, and there is a lack of knowledge about the particular molecules that make up their substrates. Gaining an insight into the specific substrates transported by these proteins might throw light on the role played by ABC transporters in CSC function and the occurrence of MDR. This in turn may lead to the development of novel strategies (Begicevic and Falasca 2017).

When MDR occurs more frequently, using chemotherapy alone becomes useless and inefficient. Thus, exploring ABC protein activity and the development of ABC transporter-targeted therapy has considerable potential to reverse chemoresistance and the ineffectiveness of cancer therapies.

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Acta Universitatis Lodziensis

Analysis of mutation occurrence in patients with acute myeloid leukaemia using next-generation sequencing

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The aim of the study was to determine the frequency of genetic changes among patients of the Holy Cross Cancer Centre in Kielce diagnosed with bone marrow cancer.

A group of 75 patients hospitalized in 2019–2021 was subjected to the study. Detection of genetic changes was performed using high-throughput next-generation sequencing, NGS, using Ion Torrent technology. The panel used detects changes associated with the development of bone marrow cancer (40 genes, 29 fusions).

The study group consisted of 75 patients diagnosed with various types of cancer. The largest group were patients with acute myeloid leukaemia, AML (n = 47); the next groups were patients with myelodysplastic syndromes, MDS (n = 14), and other myeloproliferative neoplasms (n = 14). Among all patients, 62 people had at least one mutation (83%). In the group of patients with AML, at least one mutation was observed in 46 people (98%). Among patients from the AML group, changes in 27 genes and 5 types of fusion were detected. The most frequently observed changes in the group of patients with AML were internal tandem duplication in the FLT3 gene (26%) and mutations in the nucleophosmin 1 gene, NMP1 (17%). In the group of patients with MDS, six (43%) had at least one lesion. Of the 11 types of change observed in patients with MDS, nine have been reported in patients with AML.

The results of the research revealed high genetic heterogeneity among the studied AML patients. The presence of changes in the same genes in patients from the MDS and AML groups may indicate the possibility of transformation of MDS into AML.

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Determination of the cytotoxicity of nanosilver coated with carbosilane dendrons against B14 cells

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The aim of the study was to check the cytotoxicity of silver nanoparticles (NP) coated with carbosilane dendrons – NP Ag_{143}, NP Ag_{788}, NP Ag_{1792} – and to learn more about their mechanism of action.

The methods used were the MTT assay, assessing the viability of B14 cells, and determination of the level of reactive oxygen species (ROS) using the H_2DCFDA probe. In addition, by using a JC-1 probe, the pro-apoptotic activity of NP Ag was assessed.

All tested NP Ag showed cytotoxic properties against B14 cells. Along with an increase in NP Ag concentration, a decrease in cell viability was noticeable. The most toxic of the tested compounds was NP Ag_{1792} at a concentration of 20 μM, where the cell viability was only 21% compared to NP Ag_{143} and NP Ag_{788}, for which the viability was 40% and 43%, respectively. Studies with the H_2DCFDA probe have shown that NP Ag induce oxidative stress. For NP Ag_{143}, the highest level of ROS was observed for the concentration of 10 μM and it was 4.5 times higher than that of the control. For NP Ag_{1792} at a concentration of 10 μM, the ROS content increased 4-fold. In the case of NP Ag_{788}, the highest level of ROS was observed after the action of the compound at a concentration of 5 μM (a 2.7-fold increase). The experiment with JC-1 showed that all test compounds caused a significant reduction of $\Delta\Psi m$ starting from a concentration of 10 μM. The cells incubated with NP Ag_{788} at a concentration of 15 μM for which cell viability was approx. 60% had the lowest potential value.

On the basis of the studies conducted, it can be concluded that the tested compounds are cytotoxic at higher concentrations, but the use of low concentrations of NP Ag (1–5 μM) does not cause a significant decrease in viability. The mechanism of cytotoxicity is based, inter alia, on inducing oxidative stress and generating ROS, as well as mitochondrial dysfunction.

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An analysis of the methodology for building the environmental potential of urban areas

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The United Nations (UN) predicts that until 2050 the urbanization rate in the world will be almost 70%. At the same time, the Intergovernmental Panel on Climate Change (IPCC) estimates that the average temperature on Earth will increase by 2 degrees by 2050. The effects of global warming and past harmful urban activities are visible today and include negative phenomena such as urban heat islands and urban floods. Polish Academy of Sciences specialists estimate that between 2011 and 2020 heat stress was the direct and indirect cause of nearly 28,000 annual deaths in Poland.

Today's cities exhibit low resistance to the stress caused by climate changes and low adaptation potential. It is therefore necessary to implement new solutions that will satisfy the living and existential needs of human beings, and at the same time focus on the use of natural processes. Contemporary city management should deviate from a sectoral approach to solving global warming problems and begin work on a system-evolutionary approach. It is essential to implement holistic concepts such as the blue-green network (Zalewski 2010). On this basis, effective and firm nature-based solutions should be constructed, such as rain gardens, green bus stops, and green roofs and facades of buildings. All these solutions eliminate the urban heat island effect, reduce surface runoff and improve the city's microclimate.

The aim of this study is to analyse the strategies and methods currently used to minimize the negative effects of climate change and improve the quality of life of urban residents. The latest methods of managing water resources in cities and their further development in the light of strategic UN documents will be presented.

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Radiosensitivity of human breast cancer cell line MCF-7

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Neoplastic diseases are the second most common cause of death in Poland, right after cardiovascular diseases. The most common cancer and the one with the highest mortality in women is breast cancer. Treatment of breast cancer involves surgical removal of the tumour, combined with chemotherapy and/or radiotherapy, but in addition to neoplastic changes, the margin of normal tissues is also damaged. In order to increase the effectiveness of radiotherapy, compounds are sought that would improve the effectiveness of irradiation. Recently, natural compounds of plant origin – polyphenols – have become very popular.

The aim of the research was to check whether naturally occurring compounds from the group of stilbenes (resveratrol (R), piceatannol (ROH) and piceid (RG)) affect the radiosensitivity of breast cancer cells.

The material used in the research was the oestrogen-dependent breast cancer line MCF-7. The cells were pre-incubated with stilbene derivatives at a concentration of 25 μM for 3 h, and then exposed to ionizing radiation at a dose of 2 or 6 Gy. The cytotoxicity of the compounds and of the compounds in combination with radiation was tested using the MTT assay after 24 and 72 h of incubation.

After 24 h of incubation, a statistically significant decrease was observed in all the variants used in relation to the control, while for the compound itself, a decrease was observed for ROH in combination with a dose of 2 Gy radiation and for ROH combined with both doses of 2 and 6 Gy. After 72 h of incubation, a statistically significant decrease was observed in all variants used, both for the control and for the compounds themselves.

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Combination of immunotherapy and histone deacetylase inhibitors in cancer treatment

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Nowadays, there are many methods for treating cancer. Most of them have serious drawbacks such as a lack of effective treatment for patients with metastasis, and severe side effects. The greatest hope in cancer treatment lies in the combination of different types of therapy. One such strategy is the combination of immunotherapy and histone deacetylase inhibitors (iHDACs).

Histone acetyltransferases (HATs) and histone deacetylases (HDACs) are the key enzymes that participate in the modelling of chromatin structure by attachment or detachment of acetyl groups. This process regulates gene expression. Disorders of this process can lead to cancerogenesis. Overexpression of HDACs is frequently observed in cancer cells, causing alternation of tumour suppressor gene expression. Therefore, iHDACs are a promising therapeutic tool. Furthermore, it has been proven that the use of iHDACs might lead to an increase in the efficiency of immunotherapy, a treatment strategy that uses a person's own immune system to fight cancer. iHDACs have a positive effect on immune response by increasing the immunogenicity of the tumour, which facilitates the recruitment of immune system elements. Furthermore, iHDACs increase the vulnerability of cancer cells to the effects of the immune system and reduce the immunosuppressive impact of the tumour microenvironment.

The combination of immunotherapy and iHDACs is a highly promising method of cancer treatment. Nowadays, there are many preclinical and clinical trials that focus on searching for the most beneficial combination of iHDACs with immunomodulatory factors to achieve a proper synergistic effect.

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Silver nanoparticles – possible applications and threats

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ABSTRACT

Silver is known for its biocidal properties. This metal has been used for decorations and food preservation since ancient times and has also been used in medicine. Silver foil has been used to cover wounds and burns. In addition, silver solutions were created to help fight the microorganisms responsible for causing infections, which helped the wound healing process. Currently, to increase and optimize the properties of silver, it is used on a nanometric scale. Nanosilver, due to its expanded spectrum of properties, is used in many economic sectors, including in the production of disinfectants and food films, as well as in animal farms. Nanoparticles are also the basis of nanomedicine action. Creating new drug complexes with nanosilver and modifying the medical materials used in implantology or dentistry allow the lives of many people to be saved every day. In addition, nanosilver particles are commonly used as a specific disinfectant in the production of hospital materials: dressings, bandages, surgical masks, hospital clothing and shoes, and equipment. With the growing use of nanosilver, there are concerns about its harmful effects on living organisms, because not all its mechanisms of action are known. As is well known, the dose determines the toxicity of a given substance; the case is similar for nanosilver. However, is the dose providing antibacterial and antifungal properties non-toxic to animals and humans? This review presents a summary of the scientific research showing the scope of nanosilver activity and the resulting threats.

KEYWORDS: nanoparticles, nanosilver, colloidal silver, nanomedicine, nanomaterials

Introduction

Medicine is one of the oldest and most extensive sciences, which has made enormous progress over the centuries. Development and modernization of all research and equipment as well as the progress of knowledge about all fields of medicine and methods and their practical use allow the lives of many people to be saved every day. A new multidisciplinary field of nanotechnology has found its application in medicine and pharmacy. It is a relatively new scientific discipline,

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the intensive development of which is observed in the 21st century (Rzeszutek *et al*. 2014). The precise application of nanotechnology in medicine is dealt with by nanomedicine. Its achievements have potential applications in research areas such as understanding biological processes at the molecular and cellular level, drug delivery, medical imaging, *in vitro* diagnostics, *in vivo* diagnostics, tissue regeneration, structural implants, sensory aids and surgical aids (Eaton 2007). Its activity is based on nanoparticles and nanomaterials (Song *et al*. 2019).

General characteristics of nanoparticles and nanomaterials

Nanoparticles (NP) are defined as particles of matter with a size not greater than 100 nm in each plane. This is a size comparable to that of enzymes or receptors whose size is within 5 nm. On the other hand, nanomaterials are structures in which at least one dimension is not larger than 100 nm. These include zero-dimensional structures such as quantum dots, one-dimensional structures, e.g., carbon tubes or fullerenes, and more complex structures such as dendrimers (Espinoza *et al*. 2020). Due to their nanometric size, NP have specific chemical, physical and biological properties (Sahoo *et al*. 2007). In addition to the physico-chemical properties typical of macroparticles, they gain additional unique features. They are distinguished by a high correlation of the number of surface atoms to the number of atoms in the particle core and a different behaviour under the influence of external forces (Sahoo *et al*. 2007). The unique properties obtained thanks to nanometric dimensions include disturbance of the wave function of electrons, resulting in a change of thermodynamic stability; changes in electrical, thermal and magnetic conductivity; reorganization of optical properties; increased reactivity, adsorption properties and antimicrobial activity; and agglomeration and high specific surface area (Panigrahi 2004; Su and Kang 2020).

Silver nanoparticles – properties

Scientists in nanotechnology focus their research on, among other things, metal NP. Among them, silver (Ag) is very popular. As the data show, this metal has been used since the earliest history of humankind. Silver was first used to make ornaments, but over time the spectrum of its properties has been used in everyday life for food preservation as well as in medicine. Silver foil has been used to cover wounds and burns, and Ag solutions were created to help fight the microorganisms responsible for causing infections. Currently, silver nanoparticles (NPAg) are used in many fields of science (Chen *et al*. 2009). It is also possible to modify various materials and raw materials with NPAg. This may consist of depositing NPAg in carriers or coating other surfaces with them. Thanks to such modifications, the newly created materials will acquire antifungal, antibacterial, virucidal, anti-static and impregnating properties (Xu *et al*. 2006). NPAg are observed to have from 20–15,000 element atoms in their structure. Their biocidal effect is possible due to the influence of silver on the damage to cell membranes, protein denaturation, generation of reactive oxygen species, inhibition of DNA replication and disruption of the synthesis of certain proteins (Szymański *et al*. 2012; Yamanaka *et al*. 2005). The bactericidal activity of NPAg depends on the composition of the bacterial cell wall. The presence of peptidoglycan in the cell wall reduces the sensitivity of bacteria to silver, so Gram-negative bacteria are more susceptible to the toxic effects of NP than Gram-positive bacteria (Kim

et al. 2007). Moreover, studies have been conducted which show that the formation of connections of NPAg with antibacterial drugs such as amoxicillin, penicillin G or clindamycin enhances their action (Shahverdi *et al*. 2007).

Applications of nanosilver

Disinfectants

Colloidal silver has a wide range of potential applications due to its antimicrobial properties. Despite the contemporary improvements in hygiene in biomedicine, education, the surrounding environment and industry, the problem of public health in the world is becoming important. In order to overcome various strategies, infections have been reduced by using various disinfectants (Jones *et al*. 2008). Disinfectants are chemicals that are applied to a surface to kill or inhibit microorganisms. They are useful in our daily life as they kill especially microorganisms without endangering human health. Moreover, they are abundant in quantity, efficient, cheap and non-toxic (Jones *et al*. 2008). Various chemical compounds such as alcohols, quaternary ammonium cations, aldehydes, oxidizing agents such as sodium hypochlorite, hydrogen peroxides, iodine, etc. have been successfully introduced as disinfectants; however, due to various limitations such as harmfulness, corrosivity and bacterial resistance these compounds are no longer widely used. Agents containing colloidal silver can be used for the sterilization and disinfection of both production rooms, e.g. poultry processing plants, and medical rooms. A number of tests were carried out to determine whether nanosilver can prevent the production of odorous pollutants during incubation, and thus reduce the emission of harmful gases in breeding and farm rooms. The results showed that the use of nanosilver preparations to disinfect eggs and brooders reduced microbial contamination. The bactericidal and fungicidal effectiveness of the preparation used was comparable to that of UV radiation, and its effectiveness increased during incubation. Positive results were obtained in terms of the level of organic gaseous pollutants, which decreased by 86% after the use of nanosilver preparations (Banach *et al*. 2016; Chmielowiec-Korzeniowska *et al*. 2007).

Medical rooms and hospital rooms are particularly exposed to the presence of bacteria; in order to prevent the infection of patients and staff, disinfection with nanosilver is used. One of the main routes of transmission is through contact with contaminated surfaces where nosocomial pathogens form settled communities known as biofilms. During the formation of biofilms, these pathogens are extremely resistant to antibiotics and standard cleaning procedures. Therefore, in order to eliminate the formation of biofilms on these surfaces, intense efforts have been made, especially in recent years, to develop new antimicrobial surfaces containing silver or nanosilver that can be used to prevent biofilm formation. Disinfectants in use today are less effective against certain strains of bacteria. Microorganisms such as *Staphylococcus aureus* and *Pseudomonas aeruginosa*, which are found in hospital rooms, cause chronic infections by becoming resistant to disinfectants (Khalid *et al*. 2020; McCarlie *et al*. 2020). Examples of the use of NPAg as disinfectants are shown in Table 1.

Medical equipment

Currently, colloidal silver is widely used as a disinfectant in the production of hospital materials: dressings, bandages, surgical masks, hospital clothes and shoes, and medical equipment (Leaper

Table 1. Examples of use of NPAg as disinfectants (Alonso *et al*. 2013; Close *et al*. 2016; Deshmukh *et al*. 2018; Ko *et al*. 2014; Vasile *et al*. 2017).

Material	Disinfecting activity
Ag/TiO_2	Antibacterial activity
$NPAg/SiO_2$	Fast and synergistic antimicrobial activity in air filters
Ag-co-NP	Water purification
NPAg/chitosan	Wound healing
PLA/ZnO/Cu/Ag bionanocomposites	Prolonged freshness of food products

2006). NPAg are effectively used in catheters for better antimicrobial activity and zero thrombogenicity. Cardiovascular stents and catheters require coating with antimicrobial agents such as NPAg to prevent thrombosis. NP have prolonged activity, greater bactericidal and bacteriostatic properties, and lower toxicity *in vivo* (Chaloupka *et al*. 2010).

The first cardiovascular medical device to use silver in the clinic was a silver-coated silicone prosthetic heart valve that was designed to prevent bacterial infection on the silicone valve and reduce the inflammatory response (Grunkemeier *et al*. 2006). Metallic silver can cause hypersensitivity, inhibit the normal function of fibroblasts and lead to pericranial leakage in patients (Jamieson *et al*. 2009). NPAg are safe and non-toxic in medical devices, unlike metallic silver. Therefore, Andara *et al*. (2006) synthesized a new nanocomposite with NPAg and diamond-like carbon as the surface coating of heart valves and stents, and found that the surface of the nanocomposite exhibited antithrombotic and antibacterial properties. In addition, Ghanbari *et al*. (2009) and Fu *et al*. (2006) also constructed antimicrobial multilayer films containing NPAg and investigated their *in vitro* antibacterial, mechanical and haemodynamic properties for use in coating cardiovascular implants.

Much research has been done to investigate NPAg as antimicrobial materials for coating catheters, including central venous catheters and neurosurgical catheters. Silverline (Spiegelberg GmbH and Co. KG, Hamburg, Germany) and ON-Q Silver Soaker™ (I-Flow Corporation, California, USA) are two commercially available medical catheters containing NPAg to prevent infection (Chaloupka *et al*. 2010). Medical catheters are prone to bacterial infections that can spread rapidly into the wound and its surroundings and lead to serious complications. Andara *et al*. (2006) found that nanosilver-coated plastic catheter tubes can inhibit bacterial growth *in vitro* for at least 72 hours, without significant toxicity, in an animal model.

In addition, the bone cement used in surgery is doped with NPAg with poly(methyl methacrylate) (PMMA) to reduce the risk of bacterial infections. The rate of infection has been shown to be lower with Ag and shows no cytotoxicity in murine fibroblasts or human osteoblasts, indicating good biocompatibility (Jiranek *et al*. 2006). Alt *et al*. (2004) assessed the antibacterial activity of ordinary PMMA bone cement loaded with various concentrations of NPAg *in vitro* and found that bone cement loaded with 1% nanosilver completely inhibited the multiplication of *Staphylococcus epidermidis* and *S. aureus* without a significant difference between nanosilver bone cement and the non-toxic control group and qualitative cytotoxicity tests. NPAg was also added to ultra-high molecular weight polyethy-

lene to produce inserts for total joint component replacement, and NPAg was found to drastically reduce polymer consumption (Morley *et al*. 2007). NPAg are also combined with materials such as mineral compounds or polymers, and used in implantology. This combination improves biocidal efficacy by counteracting particle aggregation, which is one of the more serious problems in the use of implants (Magalhães *et al*. 2012). In dental implantology, titanium plates are combined with nanosilver. Thanks to this application, bacteria such as *Porphyromonas gingivalis* and *Actinobacillus actinomycetemcomitans*, which cause periodontal disease, do not live on implants (Liao *et al*. 2010). In dentistry, preparations with the addition of nanosilver are increasingly used to eliminate bacterial biota. Currently, they are most often used in endodontics. There are also toothpastes on the market that contain NPAg in order to better combat oral microbiota (Pokrowiecki and Mielczarek 2012). NPAg are also used in dental appliances. It has been shown that a resin composite incorporated into materials containing NPAg exerts a long-lasting inhibitory effect against *Streptococcus mutans* (Faiyaz *et al*. 2019; Rabani *et al*. 2019; Yoshida *et al*. 1999). It has also been shown that a resin composite containing fillers implanted with silver ions releases antimicrobial silver ions on oral streptococci (Corrêa *et al*. 2015). Moreover, Magalhães *et al*. (2012) showed that the inclusion of NPAg in endodontic filling materials provides much better antibacterial activity against *Streptococcus milleri*, *Staphylococcus aureus* and *Enterococcus faecalis*. NPAg in dental adhesives is also very effective against streptococci without affecting the adhesives' mechanical properties, thus enabling their use in orthodontic procedures (Ahn *et al*. 2009; Gitipour *et al*. 2017).

Silver nanoparticle components with drugs and wound healing

The demand for drug delivery systems with a novel mode of action in order to improve the solubility and stability of potent drugs and to minimize their toxicity is a major impetus for research into drug delivery systems (Chirra *et al*. 2016; Ghosh *et al*. 2008; Mandal 2017). Features such as prolonged drug action or the release time of the active substance are also improved. Ligands can be attached to the surface of NP, allowing the distribution of drugs to precisely defined places in the biological system (Wojnicki *et al*. 2019).

The incorporation of NPAg into nucleic acid production is an ideal RNA-based therapy system. Lee *et al*. (2007) developed conjugates suitable for spherical nucleic acid colloids using NPAg functionalized with an oligonucleotide. These tricyclic disulphide groups in oligonucleotides improve particle stability and are effective in tolerating heat, ageing and oxidative degradation. The NP potential of the molecular entity to detect pathophysiological defects in malignant cells and tumours therapeutic gene load was significantly higher when it formed a drug (doxorubicin – DOX) conjugate with graphene oxide (GO). It was observed that the uptake of GO-Ag-DOX by tumour cells was 8.4 times higher than in normal cells. This GO-Ag-DOX combination not only selectively released the drug but also helped in photothermal ablation of the tumour after near-infrared stimulation (Shi *et al*. 2014). The creation of a composite of NPAg and drug resulted in increased affinity for malignant cells compared to normal cells, significantly reduced side effects and increased the chemophotothermal potential of the therapeutic agents.

Another study on the development of NPAg-drug complexes was carried out

by Chen *et al*. (2013). They developed a hybrid nanocomposite Fe_3O_4-C-Ag, the combination of which with a cytostatic, after stimulation with infrared light, resulted in increased apoptosis of neoplastic cells. Wang *et al*. (2012) developed silver nanocarriers targeting a tumour bearing the folate receptor by functionalizing the surface of NPAg with folic acid. The presence of folic acid provided NPAg with an exceptionally high affinity for tumour receptors. Nanocarrier therapy resulted in a slow release of drug (DOX) into the tumour cytoplasm and induced apoptosis. They developed the biocompatible Ag-SiO_2-mTiO_2 triplex, an excellent example of using the NP endocytosis mechanism to increase cellular uptake and consistent delivery of anticancer drugs. Cytotoxicity studies with these nanocolloids against cellular breast adenocarcinoma have shown their biocompatibility. Thanks to the use of the Ag nanocarrier, the amount of mesoporous silica increased significantly, which made it possible to deliver more drug to cancer cells. Successful *in vitro* internalization and the potential to induce apoptosis of lung adenocarcinoma cells compared to normal cells demonstrated the biocompatibility of the nanocomposite, raising the prospect of using it as a vehicle for the treatment of lung cancer (Singh *et al*. 2013).

The combination of allicin and NPAg has been tested for skin infections due to methicillin-resistant *Staphylococcus aureus* (Sharifi-Rad *et al*.). The study showed that the minimum inhibitory concentration and minimum bacterial concentration for this drug combination are lower and therefore useful in treating the skin to avoid skin infections. Silver ions contained in dressings are present at a maximum equal concentration of 1 ppm. The Ag^+ ion concentration is related to the presence of chloride ions in the wound. Silver ions react with chloride ions to form an insoluble AgCl salt, which limits the access of Ag^+ ions to the deeper layers of the wound. *In vitro* studies show that a concentration of 1 ppm is sufficient to obtain a bactericidal effect (Ip *et al*. 2006).

The participation of nanosilver in the wound healing process is related to the mechanism of local production of hydrogen peroxide and other reactive oxygen species (ROS). They are mainly produced by active NADPH oxidase in inflammatory cells. Ag^+ ions inhibit the action of serine proteases, thanks to which they have anti-inflammatory properties. Further research has proved the anti-inflammatory properties of NPAg. In a porcine model of contaminated wounds, NPAg have been shown to inhibit the activity of matrix metalloproteinases, increasing the apoptosis of inflammatory cells, thereby reducing inflammation (Chambers *et al*. 2002). Laboratory studies in mice have also provided information about the anti-inflammatory properties of NPAg. Silver ions inhibit the expression of TNF-α and IL-12 cytokines, leading to the death of inflammatory cells. However, when used at an inappropriate concentration, NPAg show anti-proliferative activity (Bhol *et al*. 2004). Therefore, it is important to determine the appropriate dose of NPAg in various types of dressings or other medical materials applied directly to wounds. However, Ag^+ ions used in the nano form can unexpectedly interact with biological systems, showing high reactivity and toxicity. The distinguishing feature of the use of nano forms is the release of 100 times more Ag+ ions than when using macro-size NPAg (Asharani *et al*. 2009).

Numerous *in vivo* studies have proven the greater antimicrobial efficacy of dressings using NPAg and not bulk silver. The penetration of xenobiotics and NP through broken skin is much easier

than through non-traumatized skin. Therefore, new dressing formulas containing silver preparations began to be developed. Studies have shown that the Ag^+ ions released are not absorbed systemically. In the case of skin burn treatment with 0.5% silver nitrate, Ag^+ ions were localized in urine and blood at a maximum concentration of 120 μg/L. It was then found that silver was deposited in the tissues, leading to argyria. Similar data were provided by a report on the condition of a patient with extensive burns to the body treated with nanosilver dressings – the boy had symptoms of argyria and the plasma silver concentration was 107 μg/kg body weight (Trop *et al*. 2006).

Food films

In recent years, there has been a growing need in the food industry to develop antimicrobial films for food packaging, bottles and containers to avoid microbial spoilage of food and to extend or preserve the shelf life of food products. Food packaging is used to protect food, vegetables and fruit against environmental pollution or bacteria, to ensure product quality and consumer safety. Oxidation and microbial invasion are the main factors causing the deterioration of product quality during production, transport and storage (Han *et al*. 2018). Currently, NPAg, silver nitrate and nanoclay are widely used in the food packaging industry to counter microbial contamination and improve barrier properties, thus extending the shelf life and freshness of packaged food and beverages (Bumbudsanpharoke *et al*. 2015; Huang *et al*. 2018; Mousavi *et al*. 2015; Tavakoli *et al*. 2017).

Colloidal silver and silver nitrate have been used in the United States for over 100 years (Nowack *et al*. 2011). Martınez-Abad *et al*. (2012) incorporated silver nitrate (0.1–10%) into ethylene-vinyl alcohol (EVOH) films and tested their antimicrobial properties against *Listeria monocytogenes* and *Salmonella* spp. They used a bacterial challenge test (Russel 2003) to evaluate the antimicrobial resistance of EVOH composite films to low-protein food samples (lettuce, apple peel and eggshell) and high-protein food samples (chicken, marinated pork and cheese) contaminated with bacterial strains. The results showed a representative number of viable *L. monocytogenes* bacteria on apple skins treated with EVOH composite membranes containing 0.1, 1 and 10 wt% $AgNO_3$ (Ag^+ ions) but in the samples coated with the composite film containing 10% $AgNO_3$ or the control ($AgNO_3$ aqueous solution), a reduction in bacterial populations was demonstrated.

In general, NPAg show a beneficial effect on the silver nitrate salt in food packaging films, since NPAg allows sustained release of Ag^+ ions due to the size-dependent Ag^+/Ag^0 ratio on their surface (Chaloupka *et al*. 2010). In this regard, low NPAg charges are added to the polymer films to release enough Ag^+ ions to ensure effective bactericidal activity (Lopez-Carballo *et al*. 2013).

Tavakoli *et al*. (2017) produced polyethylene (PE) films with 1%, 2% and 3% NPAg using an extrusion process. They proved that PE/NPAg packaging films reduce mould and *E. coli* attack on walnuts, hazelnuts, almonds and pistachios for longer periods, thus increasing shelf life and preserving nut quality. The widespread use of polymer/NPAg packaging films in the food industry has raised concerns about the migration of NPAg from films or food containers. In this context, Huang *et al*. (2011) used commercial PE/nanosilver film bags for four kinds of food-simulating solutions, representing water, acid, alcohol and fatty foods, at 25–50 °C for 3 to 15 days, respectively. Based on

spectroscopic measurements of atomic absorption, they observed the migration of Ag^0 from commercial PE/nanosilver films to food simulants. They believe that Ag^+ ions are also released from nanocomposite films when exposed to food-simulating solutions. Moreover, Ag^+ ions are readily reduced to Ag^0 in the presence of acid environments. Echegoyen and Nerin (2013) have also reported the presence of both elemental Ag^0 and Ag^+ ions in commercial polyolefin foil packages and nanosilver containers.

The European Food Safety Authority (EFSA) has recommended upper limits for silver migration from packaging, which should not exceed 0.05 mg/L in water and 0.05 mg/kg in food. It shows that determination of the silver migration level is essential to ensure strong antimicrobial activity (EFSA Scientific Committee 2011).

Food packaging falls into two categories; first, improved packaging in which nanomaterials are embedded in gas barrier plastics, and second, active packaging in which nanomaterials interact directly with food and prevent its microbial contamination. In the film-making process, NPAg are coated, absorbed or incorporated directly by a simple chemical route (Duncan 2011; Vasile *et al*. 2017). Although NPAg increase the shelf life of food, there is a need to assess the hazards and risks of their migration from the packaging to the food for consumer safety. Improved food quality and shelf life are achieved through active packaging, which reduces microbial contamination from the field, and cold storage and consumption areas (Singh and Sahareen 2017).

Cozmuta *et al*. (2015) described Ag/TiO_2 nanocomposites in packaging made of high-density polyethylene (HDP-P) film, which increase the durability and microbiological safety of bread in comparison with commonly used packaging. Orange juice kept in PE-based packaging with an $Ag\text{-}TiO_2\text{-}Fe$ composite retained the same colour, texture and taste as freshly prepared juice, even after 10 days of storage. Silver and iron have been found to have better antimicrobial properties against yeasts and moulds than TiO_2 alone (Peter *et al*. 2014).

Ramos *et al*. (2016) reported that a study of NPAg migration from a plastic baby's bottle and food container revealed less agglomeration and oxidation of NPAg. This depends on the nature of the polymer and its storage conditions. SP-ICPMS techniques were used to determine the ionic silver and NPAg in extremely diluted samples. Therefore, this method is better for obtaining accurate information on the size and concentration of NP in complex extracts in a smaller amount in a short time, avoiding agglomeration and oxidation of NPAg.

Silver and copper NP were impregnated with guar gum nanocomposites and the effects on thermo-mechanical, optical, spectral, oxygen barrier and antimicrobial properties of the film were investigated. This material showed good film properties for active food packaging applications, although the commercialization of such materials requires additional research on the effects of NP on food and further impact on human health (Arfat *et al*. 2017).

Textiles

There is also now a growing interest in the use of silver-based nanomaterials as antimicrobials in the textile sector. As sterile textiles are one of the common goals identified by scientists, bacteria-free fabrics would be subject to different uses. In order to functionalize textiles, NP can play a major role due to their specification. Various textiles properties

such as repulsion stimulation, non-creasing, anti-static, enhanced strength, hydrophobicity and antimicrobial activity are very important for increasing the durability, quality and elasticity of the textiles. The textile industry is changing and introducing new technologies not only in the processing of textiles, but also in the use of antimicrobials to avoid bacterial contamination. Nowadays, human awareness of bacterial infections caused by textile products has increased. The transfer of microorganisms from the surface of textiles to human skin is a serious health problem. Therefore, textiles can be 'treated' to avoid infection (Ahmad *et al*. 2020).

Modern methods used in the textile industry are largely based on nanotechnology. Choosing the right antimicrobial agent is a difficult task. The use of NP is more beneficial than that of traditional antimicrobial agents such as alkali metals, quaternary ammonium compounds and triclosan because they are more stable and cheaper to produce (Kim 2019; Wagener *et al*. 2016).

There are various methods of applying silver in textiles. These can involve colloidal solutions and dispersed NP as well as silver salts insoluble in water. One method is based on the reduction of the water-soluble silver salt that occurs directly on the surface and inside the pores of cellulose fibres (e.g. cotton). The product of this reaction is metallic silver at nano size. NPAg located inside the pores of cellulose fibres have the ability to aggregate, thanks to which they are fixed there, making it difficult to rinse them out of the material. This means that the enriched textile retains its bactericidal effect after repeated washing. Materials with NPAg inside and on the surface can be used for the production of medical devices used in the treatment of patients with skin infection caused by burns, wounds or postoperative infection. They can also be used to make socks, shoe inserts, underwear, duvet covers and bedding, towels, laboratory coats and medical clothing (Ilic *et al*. 2010; López *et al*. 2013).

Animal husbandry

Livestock farming is the only agricultural sector associated with animals as a source of meat, skin, milk, eggs or other foodstuffs. During the routine activity of animals, there is a possibility of infection with various pathogenic microorganisms. There is therefore a need to extend disinfection to animals. NPAg disinfectants are used to disinfect the surface of an animal's body and as a disinfectant for water. Various diseases caused by bacteria, viruses, fungi and other unicellular microorganisms have been successfully controlled with NPAg. They inhibit the reproduction and growth of bacteria and fungi responsible for infection, unpleasant odours, itching and impaired wound healing. NPAg have been found to be highly effective, fast acting, deodorizing, non-toxic, non-sensitizing, hydrophilic and therefore very effective in terms of bacterial res-istance. Therefore, NP are used as a disinfectant in animal husbandry for disinfection and disease prevention (Kovalenko *et al*. 2020; Nia 2009).

There are many potential sources of infection on poultry farms. Various microorganisms and their endotoxins are responsible for infectious diseases and spread in the environment through bioaerosols called organic dust. This organic dust reaches about 3 kilometres from its place of origin and causes serious respiratory infections (Hegarty *et al*. 2007; Tymczyna *et al*. 2007). Many attempts and studies have been made to defeat the infection in a way that does not endanger the environment and does not

have a negative impact on human health. Many chemical compounds have been used, such as organic acids, hydrogen peroxide, sodium bicarbonate, sodium orthophosphate, etc. However, none of them meets all the requirements, being variously less soluble, expensive, toxic or not able to be applied directly to the product (Hegarty *et al*. 2007). One of the potential organic compounds used for disinfection is formaldehyde due to its low cost and high biocidal activity. However, it is too toxic and carcinogenic. Data in the literature show that NP with strong biocidal properties can be an outstanding alternative (Konopka *et al*. 2009; Metak and Ajaal 2013).

NPAg are effective against a wide range of Gram-negative and Gram-positive bacteria. Gram-negative bacteria include *Acinetobacter*, *Escherichia*, *Pseudomonas*, *Salmonella* and *Vibrio*, while Gram-positive bacteria include *Bacillus*, *Clostridium*, *Enterococcus*, *Listeria*, *Staphylococcus* and *Streptococcus* (Banach *et al*. 2016). Studies have shown that NP with a diameter of 22.5 nm enhance the antimicrobial activity of some antibiotics such as penicillin G, amoxicillin, erythromycin, clindamycin and vancomycin (Anjali *et al*. 2020; Kaur *et al*. 2019; Shahverdi *et al*. 2007). Sun *et al*. (2005) found that NPAg are effective against many viruses and also inhibit HIV-1 replication. NPAg have also been found to inhibit the reproduction of a large number of fungi: *Aspergillus*, *Candida* and *Saccharomyces* (Tsai *et al*. 2019).

As a disinfectant, nanosilver plays a very important role in animal husbandry where the sanitary conditions of transport chambers or the space used for storing animals are important (Gond *et al*. 2019). Some workers have reported that diets enriched with nanomaterials reduce the toxic activity of aflatoxin-contaminated feeds (Hassan *et al*. 2019).

Sawosz *et al*. (2012) assessed the levels of NPAg residues in egg shells and tissues. This study revealed that nanosilver stimulates oxidative stress in chickens obtained from eggs disinfected with nanosilver. Disinfection turned out to be very effective in the development of embryos and makes them sensitive to even very small amounts of toxic substances.

NPAg have been tested as feed additives to stimulate the growth of birds and weaning pigs. The study found that NPAg up to 100 nm in size exhibit higher antimicrobial activity than silver salts. Silver salts are inactivated by stomach acids and easily absorbed into the body through the intestinal mucosa. At the same time, NP cannot be digested by gastric juice in the intestines and have a less toxic effect compared to silver salts (Abad-Álvaro *et al*. 2019). NPAg used as a potential dietary supplement have a positive effect on the growth of piglets, which can be achieved thanks to the antimicrobial properties of the feed. The NPAg kill bacterial groups or reduce the microbial load of the small intestine of pigs (Deshmukh *et al*. 2019). In the future, there are many opportunities to expand innovative industrial pathways using nanosilver.

Toxicity of nanosilver

The toxicity of nanosilver is closely related to the release of Ag^+ ions. The oxidation rate of these ions depends on the surface coating of nanosilver, coexisting molecules, especially thiol-containing compounds, lighting conditions and the interaction of nanosilver with nucleic acids, lipid molecules and proteins in the biological system. It has been shown that nanosilver penetrates cells and is internalized. One of the main mechanisms of toxicity is causing oxidative stress through the production of ROS, which causes DNA damage, activation

of antioxidant enzymes, depletion of antioxidant molecules (e.g. glutathione), protein binding and inactivation, as well as damage to the cell membrane (Rezvani *et al*. 2019).

Nanosilver can penetrate the cell by diffusion and endocytosis, leading to mitochondrial dysfunction. An important mechanism of the toxicity of nanosilver is its interaction with sulphur-containing macromolecules such as proteins, due to the high affinity of silver for sulphur (Guo *et al*. 2019). Arora *et al*. (2009) studied the toxicity of nanosilver in primary fibroblasts and liver cells and found that nanosilver is present in mitochondria and triggers antioxidant mechanisms. Braydich-Strolle *et al*. (2010) used mouse stem cells and found that smaller nanosilver particles were more involved in the production of ROS and the induction of apoptosis.

Trickler *et al*. (2010) found that the cytotoxicity of polyvinylpyrrolidone (PVP)-coated nanosilver in rat brain cells depends on the size and shape of the NP and causes pro-inflammatory effects. Hussain *et al*. (2005) assessed the *in vitro* toxicity of several types of NP, including nanosilver (15 and 100 nm), against a liver-derived rat cell line (BRL 3A). After 24 hours of exposure, mitochondrial function and membrane integrity (measured as leakage of lactate dehydrogenase) were significantly reduced at the 5 and 10 mg/mL doses. The leakage of lactate dehydrogenase was dose-dependent and greater for 100 nm NP than for 15 nm nanosilver.

The results of many studies indicate that there are several routes of exposure to the toxic effects of nanosilver (Figure 1). Exposure to nanosilver can lead to genotoxicity and DNA damage (Ivask *et al*. 2015; Lebedová *et al*. 2017; Li *et al*. 2017b), an inflammatory response in the liver and kidneys, and lung, heart, intestinal and spleen dysfunction (Lankveld *et al*. 2010; Rosas-Hernández *et al*. 2009). Numerous in vivo studies on mammalian cells suggest that the liver is particularly vulnerable to nanosilver exposure, which, due to its detoxifying role in the body, accumulates relatively

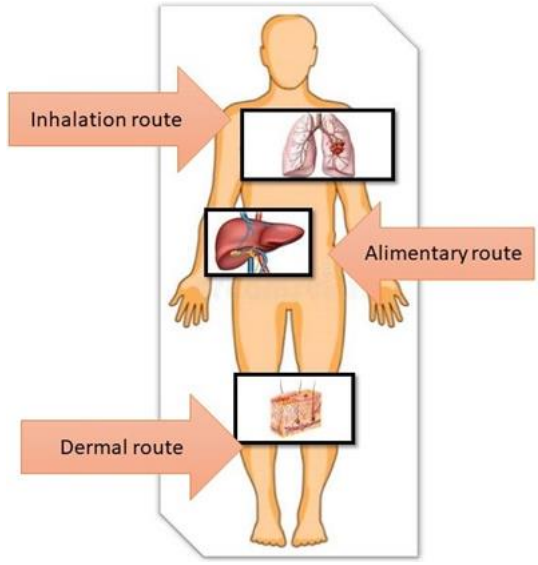

Figure 1. Potential routes of exposure to the toxic effects of nanosilver

large amounts of NP (Ema *et al*. 2017; Jia *et al*. 2017). Recent proteomic research has proven the adverse health effects caused by exposure to Ag^+ and NPAg, where both forms of silver induced similar forms of signalling and metabolic changes (Juling *et al*. 2018). Subsequent work on the toxicological effects of NPAg and Au reports increased liver enzyme function, indicating liver toxicity and damage. Hepatitis is manifested by an increase in the amount of t he inflammatory cytokines interleu-kin-6 (IL-6) and tumour necrosis factor alpha (TNFα) (Al-Bishri 2018).

The increasing possibilities of using nanosilver contribute to the increase in the number of people working in exposure to this substance; therefore, it is important to understand the mechanism of toxicity of NP to human cells. In studies on the toxic effects of NPAg on human cells, it was shown that, like in mice, the liver is the organ most susceptible to NPAg accumulation. The effect of NPAg on the HepG2 cell line was checked using the micronucleus test, viability test and DNA array analysis. Research shows that many major biological processes are altered, ultimately leading to cell apoptosis (Cavallin *et al*. 2018; Sahu *et al*. 2015). Genotoxicity is based on the evolution of the major DNA damage response pathway. The GADD45a gene was tested after exposure to NPAg in HepG2 liver and A549 lung epithelial cells. The results showed that NPAg produces a strong dose-dependent transcriptional increase and activation of the GADD45a promoter. This is indicated by luciferase activity along with a significant decrease in cell viability. Additionally, compared to A549 luciferase cells, HepG2 luciferase cells are more susceptible to NPAg because a higher level of genotoxicity is induced (Wang *et al*. 2017).

Inhalation is one of the potential ways of being exposed to nanosilver, particularly through consumer products such as disinfectants in the form of sprays. Inhaled NP can accumulate deep in the lungs and interact with pulmonary surfactant (PS). It has been found that upon contact with PS, NPAg are immediately surrounded by a crown of biomolecules that contains both lipids and proteins. While lipids remain unchanged, proteins undergo significant changes (Hu *et al*. 2017). Toxicity studies of starch-coated NPAg against IMR-90 human lung fibroblasts showed a dose-dependent reduction in ATP con-tent and DNA damage due to Ag deposition and interaction with DNA followed by G2/M cell cycle arrest (Asharani *et al*. 2009). Studies by Gliga *et al*. (2018) on the BEAS-2B cell line involved exposing the cells to 1 mg/mL NPAg (10 nm) for 6 weeks. RNA sequencing, as well as genome-wide DNA methylation analysis, showed that repeated, long-term human exposure to low doses of nanosilver is pro-fibrotic and induces epithelial-mesenchymal transition (EMT) and cell transformation.

Subsequent studies on the cytotoxicity of modified PEI-NPAg (polyethyleneimine-silver nanoparticles) and PEG-NPAg (polyethylene glycol-silver nanoparticles) were carried out on HTB182 lung cancer cells and normal human bronchial epithelial (hBE) cells. NPAg toxicity to HTB182 and hBE was shown to be dose-dependent and more noticeable in cancer cells. The surface modification of NPAg significantly influenced their anticancer/anti-proli-ferative properties (Su *et al*. 2017).

NPAg can now easily come into contact with the human reproductive system through the use of contraceptives and hygiene products. Given that NPAg can cross the placenta and that pregnant women and the early fetus are more

susceptible to harmful external factors, it is important to understand the effects of nanosilver on the reproductive system and fetal development. The studies of (Kang and Park 2018) found that the effect of the minimum amount of Ag^+ ions on human prostate cancer cells reduces the transactivation of dihydrotestosterone, an essential male reproducetive hormone (Ema *et al*. 2017). Another relatively new study on sperm functionnality showed that NPAg adversely affect genes involved in spermatogenesis and sperm functionality, at both low and high doses. This study showed that NPAg interfere with the chemical activity of the reproductive endocrine system in prepuberty and adolescence (Cavallin *et al*. 2018).

Nanosilver is present in many consumer products, the production, use and disposal of which can lead to environmental hazards. Disinfecting and washing products are one of the many ways that NPAg can enter the ecosystem. They undergo many transformations in the environment, including aggregation and agglomeration, the most significant of which are dissolution and the resulting formation of various chemical compounds, mainly sulphides and chlorides. Silver sulphide (Ag_2S) is important because it is insoluble in all solvents, making it a persistent compound in the environment (Li *et al*. 2017a). Ag_2S can be found in wastewater treatment plants and sometimes even in freshwater. The biotoxicity of silver depends directly on the type of silver compound present in the environment. While the movement of Ag^+ in soil and sediments is extremely limited, silver may behave differently on a nanometric scale (Levard *et al*. 2012).

Summary

This article provides a comprehensive and up-to-date overview of the synthesis and properties of NPAg and their antibacterial and cytotoxic effects in mammalian cells. The bactericidal activity of NPAg has led to their widespread use in cosmetics, medical products, anti-microbial dressings, etc. However, the extensive use of NPAg has raised serious public concerns regarding the safety and environmental impact of these products. In this regard, it is considered necessary to study the interaction between NPAg and biological cells in order to better understand the health risks of using NP. Many studies have shown that nanosilver damages membranes and is responsible for mitochondrial disorders, the generation of ROS, oxidative stress and DNA damage.

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Buffer composition affects rose bengal dialysis rate through cellulose membrane

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ABSTRACT

Due to its fluorescent and phototoxic properties, rose bengal (RB) is used in photodynamic therapy. To improve the delivery of RB to its site of action, the application of nanocarrier systems has been proposed. The most promising approach includes the use of pH-responsive nanoparticles. To evaluate the pattern of drug release in different buffers, equilibrium dialysis is commonly used. Here, we used water and two buffers to determine the impact of solvent composition on the aggregation and dialysis rate of RB through a cellulose membrane. The results show that buffer composition does not influence the fluorescent properties of RB. However, the presence of additional ions causes a change in diffusion rate that is most probably linked to the size of RB aggregates.

KEYWORDS: nanocarriers, dialysis, rose bengal, photodynamic therapy.

Introduction

Rose bengal (RB) is one of the synthetic dyes, designed as a fluorescein analogue and classified as a xanthene (Snyder and Paugh 1998). It is used in the textile (Alexander 2010), food (Cossu *et al*. 2016) and pharmaceutical industries (Patel *et al*. 2020). In medicine, RB is used for staining damaged conjunctival and corneal cells to indicate eye damage (Bron *et al*. 2003) and for diagnosis of liver and eye cancer (Capinera and Squitier 2000) as well as (due to the fluorescent properties) as a photosensitizer in anticancer photodynamic therapy (Qin *et al*. 2017). RB is characterized by maximum absorbance at 550 nm, high efficiency of free radical production, fluorescence emission, low photodegradation, non-toxicity in the dark and a lack of allergic reactions and other side effects (Allison *et al*. 2004). However, its applicability may be limited due to the hydrophilic nature of RB, leading to its poor cellular uptake (Gianotti *et al*. 2014). Thus, the main advantage of nanotechnology in this case is based on increasing transmembrane transport of the drug, leading to an increase of its photodynamic potential (Sztandera *et al.* 2020). Nowadays nanotechnology

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allows the synthesis of complex nanocarriers such as dendrimers (Dabrzalska *et al*. 2017), polymersomes (Villani *et al*. 2017) or dendrimersomes (Apartsin *et al*. 2020). Depending on the type of nanoparticle, RB can be conjugated via a linker to the surface of the nanoparticle or encapsulated inside its structure (Patri *et al*. 2005). To limit potential side effects, a photosensitizer should be released from its nanocarrier under strictly controlled conditions occurring only in neoplastic lesions. pH is probably the best factor for triggering the release in the case of anticancer therapies, due to the slightly acidic tumour environment (Sztandera *et al*. 2019). Evaluating this feature is crucial during the design of new nanosystems. For these assays and for the purpose of purification steps, in which free RB is removed from the nanocarrier solution, equilibrium dialysis is most frequently used. Application of semi-permeable membranes with an optimal weight cut-off allows the separation of molecules with different sizes, and the use of different pH conditions enables an evaluation of pH-dependent release of the drug from its nanocarrier. However, since different nanoparticles and carrier systems require different buffers, which may affect the properties of the therapeutics and dialysis membranes, it is important to evaluate the correlation between buffer composition and the rate of RB dialysis through the membrane.

Materials and methods

Materials

RB and PBS were purchased from Sigma-Aldrich (Taufkirchen, Germany). Snakeskin™ Dialysis Tubing (3.5K MWCO, 22 mm) was obtained from Thermo Fisher Scientific (Waltham, MA, USA). Na_2HPO_4 and NaH_2PO_4 required to prepare phosphate buffer were purchased from POCH (Gliwice, Poland).

Spectroscopy studies

Fluorescence spectra were acquired using a PerkinElmer LS-50B spectrofluorometer. Measurements of the evaluated RB samples were performed in proper buffer, at room temperature. The excitation wavelength was set to 525 nm and spectra were collected in a wavelength range from 540 to 640 nm. Excitation and emission slits were 5 and 7 nm, respectively.

Dialysis

To evaluate the rate of photosensitizer dialysis against different buffers, a 50 μM solution of RB was enclosed in dialysis membrane tubing (SnakeSkin™ Dialysis Tubing, 3.5K MWCO, 22 mm; Thermo Fisher) and immersed in PBS (10 mM, pH 7.4), phosphate buffer (10 mM, pH 7.4) or H_2O. Samples from the internal phase were collected at subsequent intervals (0.5, 1, 2, 4, 8 and 24 h) and measured spectrophotometrically using a PerkinElmer LS-50B spectrofluorometer.

Size measurements

Size measurements were performed using a Zetasizer Nano ZS (Malvern Instruments Ltd., UK) at a constant temperature of 25 °C to check the hydrodynamic diameter of RB immersed in different buffers. Samples at the final concentration of RB (100 μM) were prepared in PBS (10 mM, pH 7.4), phosphate buffer (10 mM, pH 7.4) and H_2O.

Statistical analysis

Two-way ANOVA for concentration series followed by post-hoc Tukey's test for pairwise difference testing were used to test statistical significance. In all tests, p-values < 0.05 were considered statistically significant. Data are presented as arithmetic mean ± SD.

Results

Fluorescence measurements showed that the maximum fluorescence intensity of RB occurs at 565 nm regardless of the buffer used (Figure 1). Moreover, the fluorescence intensity of RB was almost the same in all examined buffers.

Based on the fluorescence intensity from the previous experiment, we calculated the percentage of RB remaining in the dialysis tube after a specified time (Figure 2). RB diffused through the cellulose membrane over time; however, the release rate was dependent on the type of external buffer. Dialysis against PBS was the most rapid – after 6 h almost all RB molecules were released. Diffusion in phosphate buffer was slower; however, the release rate after 24 h was like that in PBS. By contrast, dialysis against water was very slow and after 24 h only 20% of RB was released from the dialysis tube.

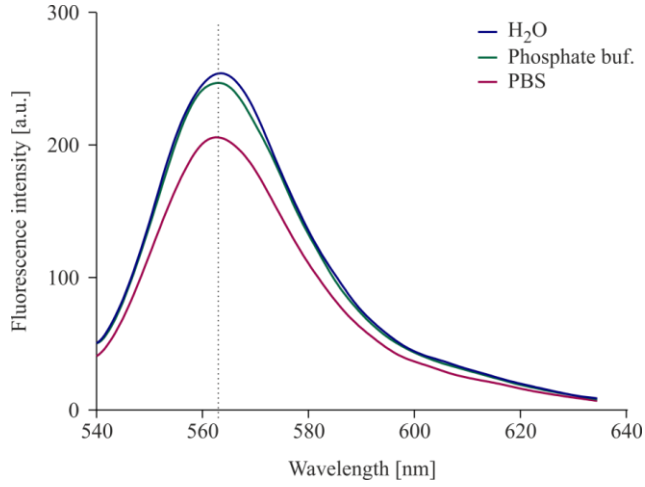

Figure 1. Fluorescence spectra of RB (1 µM) in PBS, H_2O and phosphate buffer.

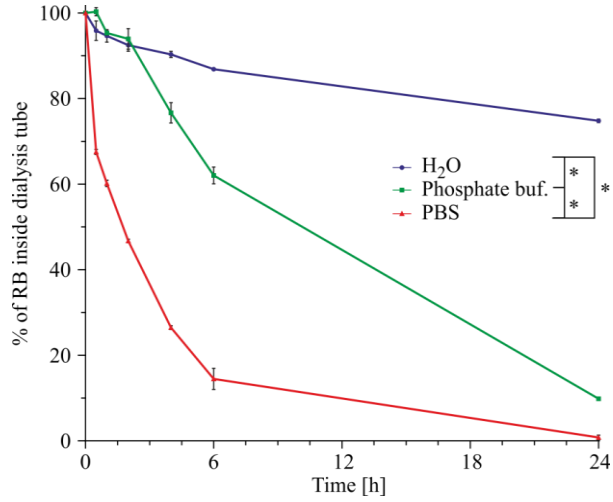

Figure 2. Dialysis of RB against different buffers. Data presented as mean ± SD, *p < 0.05.

Table 1. Hydrodynamic diameter of RB dissolved in evaluated buffers.

	H_2O	Phosphate buffer	PBS
Hydrodynamic diameter [nm]	120.76 ± 37.25	1.27 ± 0.32	0.72 ± 0.13

Measurements of hydrodynamic diameter showed that the composition of the buffer influences the aggregation of RB. The largest RB aggregates were observed in H_2O, while in PBS and phosphate buffer there were almost no clumps observed.

Discussion

Equilibrium dialysis is a relatively easy but very important step in the preparation and characterization of new drugs and their carriers. Owing to the application of semi-permeable membranes, this method allows the purification of final products from residues and checking of the conditions of drug release from nanosystems (Apartsin *et al*. 2020; Wu *et al*. 2013). Our experiments have shown that buffer composition does not cause a shift of the maximum emission wavelength of RB, but greatly impacts the diffusion rate of this dye through semi-permeable cellulose membrane. Dialysis buffers used in the experiments differed in their ion content: water was ion-free, phosphate buffer contained sodium and phosphate ions, and PBS in addition to these also contained chloride and potassium ions in high concentrations (Cold Spring Harbor Protocols 2006). In the case of dialysis, buffers have an additional advantage as opposed to water – they are able to maintain a constant pH, which is critical in the case of pH-sensitive nanosystems (Karimi *et al*. 2016). Based on hydrodynamic diameter measurements, we can conclude that the composition of buffer influences RB aggregation, which results in the presence of RB in the solution in the form of aggregates of various sizes (largest in water, smallest in PBS buffer, most probably due to interactions of the anionic RB with oppositely charged ions in the solution). A cellulose membrane has a so-called molecular weight cut-off (MWCO), which is the largest particle size that can pass through the membrane. Thus, smaller molecules diffuse more rapidly than particles with a size close to the MWCO, which is reflected in our results – the aggregates in water could not cross the membrane as fast as those in phosphate buffers. Moreover, in a production process, a membrane with heterogenous pores is usually obtained, which may additionally slow down the dialysis rate (Haney *et al*. 2013). Finally, one additional possible explanation involves changing the properties of hydrophilic cellulose membrane in the presence of counter-ions and facilitating the passage of solutes.

Conclusions

Buffer composition significantly influences the diffusion of RB through a semi-permeable cellulose membrane. The most probable explanation involves the influence of ions contained in the buffers that protect RB from clumping compared to the environment of ion-free water, where the biggest aggregates were observed. Increased size of RB aggregates limits dialysis efficiency. Thus, the optimization of this method is crucial to prevent miscalculation of the concentration of final product or misstatement about the pH-dependent stability/lability of bonding with nanoparticles.

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A brief dive into the phenomenon of cisplatin resistance in non-small-cell lung cancer

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ABSTRACT

Lung cancer is one of the most lethal types of cancer due to a lack of proper treatment. The rare presence of molecular therapy targets forces the use of platinum-based drugs. Cisplatin, approved by the USA as an anticancer therapy in the 1970s, is still one of the most prominent therapies against lung cancer. Unfortunately, the biggest limitation of cisplatin-based therapy is the development of cisplatin resistance. Cancer cells overcome the vast DNA damage caused by the drug in a variety of ways such as detoxication and extracellular transport of the drug, enhanced repair mechanisms, omitting apoptosis and epigenetic alterations. Chemotherapy resistance is an issue that so far cannot be dealt with. Nevertheless, better understanding of the molecular pathways behind cisplatin resistance brings hope for better therapy outcomes in lung cancer patients.

KEYWORDS: non-small-cell lung cancer, cisplatin, cisplatin resistance

Introduction

Despite the decades of extensive studies on cancer, it remains one of the leading causes of human deaths worldwide. It is estimated that in the United States of America in 2021, over 1.8 million new cancer cases will be diagnosed, while over 608,000 Americans will die from a variety of cancer types. It is expected that almost 25% of cancer-related deaths in both women and men will be caused by lung cancer. Due to that fact, lung cancer is placed among the most lethal cancer types in both sexes (Siegel *et al*. 2021). Although several causes of lung cancer are listed in the literature, tobacco smoking is considered a critical factor for the development of lung cancer. A variety of cancerogenic substances in the tobacco smoke may cause DNA damage, which in turn may cause mutations and therefore lead to cancer initiation and progression (Dela Cruz *et al*. 2011).

Lung cancer can be divided into two subtypes: small-cell lung cancer (SCLC) and non-small-cell lung cancer (NSCLC)

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(Zappa and Mousa 2016). Unfortunately, most NSCLC cases (85–90%) lack defined molecular targets for targeted therapies; therefore, platinum-based chemotherapeutics are widely used in NSCLC treatment (Fennell *et al*. 2016).

The commonly known platinum-containing compound cisplatin is frequently used in the approach to cure multiple types of cancer. Due to its alkylating properties, cisplatin prevents DNA strands from uncoiling by forming adducts with guanine nucleotides, which in turn debilitates DNA replication (Wishart *et al*. 2018). In response to cisplatin-related DNA damage, DNA lesion repair machinery is triggered. Therefore, proteins engaged into mechanisms such as nucleotide excision repair (NER) or mismatch repair (MMR) attempt to restore DNA integrity. Depending on the severity of the DNA damage, cells may either undergo cell cycle arrest and repair the DNA, or proceed to cell death (Figure 1) (Galluzzi *et al*. 2012). Although cisplatin has been proven to be at least to some extent successful as an anticancer therapy, there are several limitations to its use. In addition causing to nausea, cisplatin is known to be nephro- and neurotoxic (Fennell *et al*. 2016).

The problem of cisplatin resistance

Possibly the greatest limitation of successful use, not only of cisplatin but many other chemotherapeutics as well, is the development of drug resistance by cancer cells. Cisplatin resistance is a complex phenomenon that integrates a variety of molecular processes in order to ensure cell survival. Cancer cells are able to adapt to some extent to the presence of the drug and overcome its activity by, for example, enzymatic deactivation or a change in the activity of the drug, drug efflux, extensive lesion repair or alterations in epigenetics (Figure 2) (Gąsiorkiewicz *et al*. 2021). The aforementioned strategies will be briefly addressed in the following paragraphs.

Glutathione-dependent resistance

Glutathione (GSH) plays a variety of roles in mammalian cells. Its activity includes inter alia protection from reactive oxygen and nitrogen species, and detoxification of various compounds as

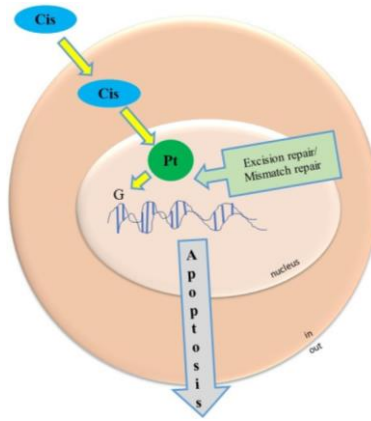

Figure 1. General mechanism of cisplatin response. Cisplatin forms DNA adducts with guanine bases in DNA. Depending on the severity of the DNA lesions, cells may repair the DNA using NER or MMR pathways, or undergo apoptosis. Cisplatin is depicted by blue ellipses and the platinum atom by a green ball.

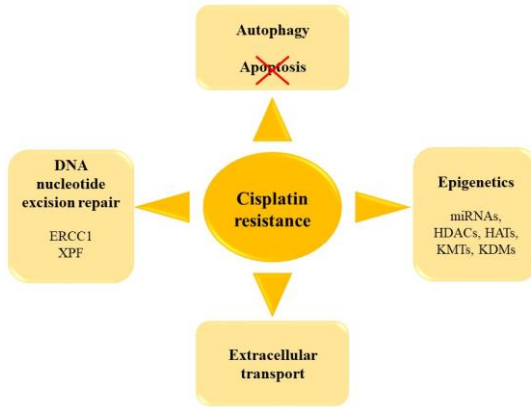

Figure 2. Main cisplatin resistance mechanisms in lung cancer.

well as extracellular transport of harmful agents (Pizzorno 2014). The latter function of GSH is facilitated by glutathione-S-transferase (GST), which promotes the formation of GSH-drug complexes. Next, the complexes are removed from the cells via ATP-binding cassette transporters (ABC transporters) such as ABCC1, ABCC2 and ABCB1 (Lan *et al.* 2018). While most of the efficiency in counteracting cisplatin takes place due to drug efflux, some of its activity to form DNA adducts is diminished by the formation of GSH-drug complex. Increased efficacy of complex formation has been observed upon increased expression of the P1-1 variant of GST (Peklak-Scott *et al.* 2008).

Autophagy-related cisplatin resistance

A variety of cell-stressing factors including cisplatin may lead to the autophagy of cancer cells. Autophagy allows a cell to recycle energy extracted from digesting its own components in order to cope with stressors. As autophagy is considered an anti-chemotherapeutic measure, cisplatin-resistant cells tend to exhibit a high level of autophagy (Gąsiorkiewicz *et al.* 2021), which suggests the potential involvement of autophagy in the origin of cisplatin resistance. Additionally, autophagy can be induced by hypoxic conditions. It is a common condition for solid tumours to lack a proper oxygen supply due to insufficient vascularization of the tumour site; therefore, those starving cancer cells redirect their metabolism into autophagy (Yun and Lee 2018). Hypoxia-induced metabolic and genetic changes desensitize cancer cells to cisplatin treatment in comparison to cells with a proper oxygen supply. Under hypoxic conditions, cancer cells are less likely to undergo apoptosis due to inhibition of Bax protein translocation to the mitochondria. Moreover, under hypoxic conditions, expression of the pro-apoptotic mediators BNIP3 and BNIP3L is reduced in cisplatin-treated lung cancer cells. The opposite effect is observed when cells are subjected to either hypoxia or cisplatin alone. Additionally, the simultaneous presence of hypoxia and cisplatin treatment robustly elevates expression of the autophagy markers Beclin-1, p-Beclin-1, LC3-II and p65 (Wu *et al.* 2015). Therefore, hypoxia-induced autophagy may support cisplatin resistance by redirecting cancer cells from DNA damage-triggered apoptosis into autophagy that supports cancer cell survival.

Nucleotide excision repair in cisplatin-resistant cells

As mentioned previously, NER is the main DNA damage repair mechanism involved in dealing with cisplatin-induced DNA adducts. Although the exact mechanism by which the NER pathway influences cisplatin resistance remains unknown, some proteins involved in the process have been identified. Two components of the NER machinery, endonuclease XPF accompanied by ERCC1 protein, have been acknowledged to enhance cisplatin-induced DNA damage in drug-resistant cancer cells (Rocha *et al*. 2018). Knockdown of either of the proteins results in increased cisplatin toxicity in NSCLC cells. Moreover, simultaneous knockdown of both proteins results in a cumulative cytotoxic effect (Arora *et al*. 2010). Notably, histone deacetylases (HDACs) may be involved in the regulation of ERCC1 expression in lung cancer. The introduction of HDAC inhibitors (iHDACs) increases the acetylation of E2F1, which promotes association of E2F1 with the promoter region of miR-149. The miRNA recognizes and binds the 3' UTR region of ERCC1, decreasing its expression and thus promoting cisplatin toxicity in NSCLC cells (He *et al*. 2020).

Epigenetic alterations in cisplatin resistance

Histone-modifying enzymes, such as histone acetyltransferases (HATs), deacetylases (HDACs), methyltransferases (KMTs) and demethylases (KDMs), play a role in NSCLC development. In particular, some of these enzymes have been involved in the development of cisplatin resistance: BRCA1/2, HDAC6, KAT5, KAT3A, KAT2B, KAT13B, KAT13D, KMT6 (O'Byrne *et al*. 2011). For example, ubiquitin-specific peptidase 10 (USP10) has been found to stabilize HDAC6, thus promoting its hyperactivity. Moreover, both enzymes are overexpressed in lung cancer. USP10 knockdown leads to increased cisplatin toxicity in a p53-deficient murine xenograft model (Hu *et al*. 2020). Besides epigenetic writers and erasers, miRNAs play a variety of regulatory roles crucial for tumour progression and survival as well as in the development of cisplatin resistance (Fadejeva *et al*. 2017). miR-29c expression is decreased in tumour samples taken from NSCLC patients. Interestingly, the miRNA acts as a cisplatin resistance suppressor, by specific inhibition of AKT2, a key signal transducer in the PI3K/AKT pathway (Sun *et al*. 2018).

Conclusions

The phenomenon of cisplatin resistance or chemotherapy resistance in general is a complicated issue. Despite years of extensive studies, the problem remains elusive and successful anticancer therapy is still non-existent. Besides the off-target toxicity, the main problem of chemotherapy resistance is the multitude of potential cellular pathways and mechanisms that may substitute those that are targeted and inhibited with specific compounds. Nonetheless, the better the understanding of the molecular mechanisms behind cisplatin resistance, the better the potential outcomes and survival chances for cancer patients.

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Challenges and development directions of membrane bioreactors operated on passenger ships in international shipping

BIOOPEN 2021 – POST-CONFERENCE ARTICLE

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ABSTRACT

In membrane bioreactor (MBR) technology, the activated sludge method is integrated with the separation of solid particles by ultrafiltration (UF). The technology ensures a high effluent quality, a shortened hydraulic retention time and a long sludge age that promotes slowly growing microorganisms and low sludge production. These advantages and the modular construction mean that MBRs have started to treat wastewater generated on passenger ships to adjust the treatment systems to the International Convention for the Prevention of Pollution from Ships. The aim of this paper is to present operational aspects of MBRs treating wastewater generated on ships, which are different from the aspects of MBR operation on land. This paper describes the consequences of separate treatment of gray wastewater (from showers, washing machines and kitchens) and black wastewater (from toilets), and of discontinuous flow of wastewater resulting from very high variability in the passenger number and the use of the MBR as a ship ballast element. The possibility of introducing a water recovery technology using the existing infrastructure on passenger ships as well as the hybrid UF/reverse osmosis technology is presented. The findings demonstrated that gray effluent may be reused for marine main engine cooling jackets of high and low temperature, ship boilers or ship laundry.

KEYWORDS: ultrafiltration, gray wastewater, black wastewater, water reuse

Introduction

In marine bioreactor (MBR) technology, wastewater treatment with the activated sludge method is integrated with the separation of solids in the membrane ultrafiltration (UF) module, which due to its function corresponds to the secondary settling tank. As a result of UF, permeate is released, directed to the treated wastewater tank, and retentate, that is, thickened activated sludge, is recirculated to the activated sludge chamber (Chiemchaisri *et al.* 1993,

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Goosen *et al*. 2005, Laurinonyte *et al*. 2017).

Compared to conventional systems with a secondary settling tank, the MBR technology has the following advantages: a high quality of treated wastewater, providing the basis for water renewal; a high concentration of biomass in the reactor, which allows shortening the wastewater retention time and even a threefold reduction in the cubature of the activated sludge chambers; a long sludge age that favours the growth of slowly growing microorganisms; and low excess sludge production, disinfection of wastewater, elimination of the secondary sedimentation tank, modular arrangement of membranes and reduction of the area required for the construction of the wastewater treatment plant (Chua *et al*. 2002, Lin *et al*. 2012, Fazal *et al*. 2015). The disadvantages of the system include contamination of membranes (fouling) and the requirement of a high aeration intensity.

The advantages of the MBR technology put it at the level of best available techniques (BAT) regarding wastewater treatment. The modular nature of an MBR system and other benefits of this technology have led to MBRs being used to treat wastewater from marine facilities. This use is related to the necessity to adjust the wastewater treatment systems to the applicable legal regulations.

Knowledge of the MBR technology is mainly related to onshore installations. The operation of MBRs on ships has distinct conditions compared with onshore systems, namely the separation of wastewater generated on the ship into black wastewater and gray wastewater. Separate treatment of gray and black wastewater streams with a highly efficient technology increases the flexibility of the system and the recycling potential of gray wastewater, which after treatment can be used, for example, for laundry. The efficient management of these separate streams and the ability to recover water are limited by another feature of the ship's installation: the discontinuity of the wastewater flow. The lack of continuity results from great variability in the number of people using the ship as well as from the fact that the MBR is used as a ballast element of the ship. Depending on the priority needs of maintaining stability, gray wastewater and permeate are kept in ballast tanks or released; hence, there is marked variability in the hydraulic load of the MBR installation, even leading to a shutdown of the installation. The objective of this paper is to indicate these operational aspects of MBRs used to treat wastewater generated on ships that distinguish them from onshore MBR systems.

Legal regulations

Wastewater and sludge management on board a ship is strictly regulated. The basic legal act regulating the discharge of wastewater into sea waters is the International Convention for the Prevention of Pollution from Ships (MARPOL), with its annexes and directives of the Maritime Environmental Protection Committee (MEPC) (MARPOL 73/78 1973/1978, MEPC.227(64) 2012). In addition, MARPOL regulates the provisions on special sea areas and particularly sensitive sea areas. For example, the Baltic Sea and the Baltic States are a special area with a categorical prohibition on the discharge of wastewater, regardless of the ship's location. Normal areas (except special areas) are open sea areas, outside territorial waters, more than 12 nautical miles from the coastline (MARPOL 73/78 1973/1978, MEPC.227(64) 2012).

Annex 4 to MARPOL, introduced in 2003, states that outside of special areas (MARPOL 73/78 1973/1978):

– comminuted and disinfected wastewater (gray wastewater) may be discharged into the sea at a distance of more than 3 nautical miles from the coast,

– non-comminuted and non-disinfected wastewater (black wastewater) and excess sediment can be discharged into the sea at a distance of more than 12 nautical miles from the coast,

– in any event, the vessel must be traveling at a speed greater than 4 knots,

– irrespective of the ship's location, the wastewater treated in an efficient wastewater treatment plant can be discharged, as long as the wastewater will not cause visible solid particles to float in the water or change the colour of the water.

Annex 4 to MARPOL states that in special areas the discharge of wastewater is prohibited unless there is an efficient wastewater treatment plant on board the ship and the treated wastewater does not cause visible solid particles to float in the water or change the colour of the water. Moreover, additional manufacturer's requirements specified in the technical and operational documentation stipulate that the MBR installation may operate in special, particularly sensitive areas, as well as at a distance of less than 12 nautical miles, provided that the activated sludge is removed in the amount of 10% of the current content of the activated sludge chambers on the day of the planned stay in this zone, which is illustrated in the formula:

$$10\% \times m^3 \times d = n[m^3]$$

This means that depending on the number of days the ship spends in a given area, meeting the requirements of the installation manufacturer will have an impact on the operation of the MBR installation, for example, during a planned 14-day cruise on the Baltic Sea, where there is a total ban on discharge.

The presented requirements result in the necessity to adapt the MBR system operation to the geographical location of the ship, the number of days the ship stays in special areas, the variable load of the wastewater treatment plant with the load of pollutants resulting from different characteristics of gray and black wastewater and the variable hydraulic load of the installation resulting from the number of people and the use of wastewater for ballasting the ship.

Description of the example installation

The issues of the installation as well as the operational aspects have been described using the example of the real object of the advance wastewater treatment systems (MBR), which is operated on a ship that carries 100–3000 passengers.

The MBR installation (Figure 1) is adapted to work in three modes: with black wastewater, gray wastewater or a combination of both. There are two identical MBR installations on the ship. The discussed configuration is the only one of this type with dedicated treatment plants: the starboard MBR purifies gray wastewater and the port MBR purifies a mixture of gray and black wastewater. This solution increases ecological safety. Biological wastewater treatment is carried out in anoxic and aerobic chambers. The system also consists of a 500 m^3 gray wastewater retention tank and four wastewater and ballast tanks with a volume of 500 m^3 each. These tanks also receive permeate from the treated wastewater tank when the ship is in an area where discharge of wastewater is prohibited. The important fact that these tanks are used for ballasting the ship affects the hydraulic load of the MBR installation and the share of black

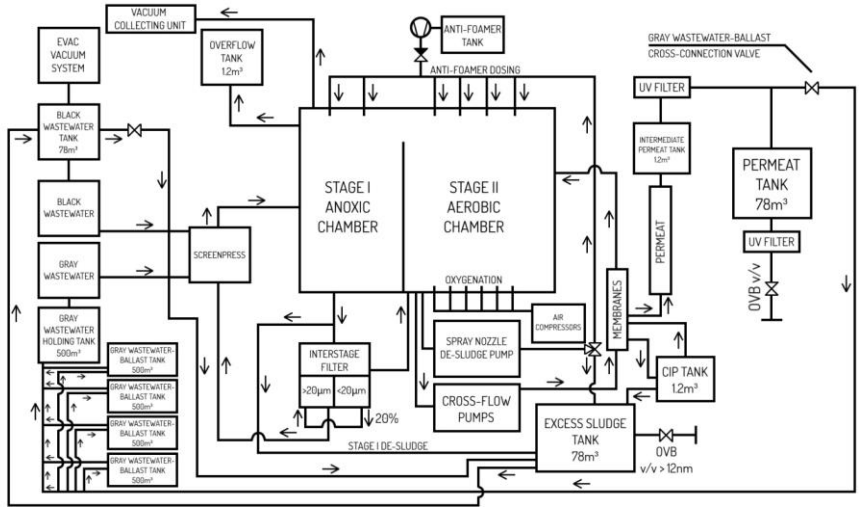

Figure 1. Scheme of the marine bioreactor (MBR) installation.

wastewater in the inflow to the installation. The share of black waste-water, in turn, influences the work of the interstage filter (ISF), which is a centrifugal separator with a capacity of 100 m^3/h. Wastewater and activated sludge from the anoxic chamber are directed to the oxygen chamber through this filter. The ISF separates solids from liquids: wastewater with a solid particle size > 20 µm returns to the anoxic chamber and particles < 20 µm go to the aerobic chamber. Wastewater with solids > 20 µm can be diluted by wastewater with solids < 20 µm to improve treatment. Optimisation of the waste-water flow procedure through the ISF chambers is important in the situation of a large share of black wastewater (70%) with a high concentration of total suspended solids (TSS) of 200–500 mg/l in the inflow to the reactor. Moreover, the separation of particles on the ISF influences the size of the load of organic compounds in the wastewater introduced into the aerobic chamber, which deter-mines the nitrification. The membrane module with UF membranes is supplied from the activated sludge oxygen chamber. The permeate goes to the 1.2 m^3 treated wastewater tank, then to the 78 m^3 treated wastewater tank and is discharged overboard or directed to the wastewater-ballast system, depending on the ship's position and speed. Retentate – concentrated activated sludge – returns to the aerobic chamber. The number of days the ship is in special areas and the hydraulic load of the system affect the flow through the membranes and carry the risk of the membranes going into stand-by, causing the membranes to foul and reducing their hydraulic performance. The membranes are also equipped with a clean-in-place (CIP) system that includes 1.2 m^3 membrane washing tank fed with service water that is produced by evaporators or reverse osmosis without using mineralisers. This water is used for closed-loop chemical rinsing as well as for backwashing, where each programmed open-loop cycle removes contaminants into the sludge tank and fills the rinsing tank once again. According to legal regulations, the excess sludge tank that collects excess sludge from the anoxic and aerobic chambers and technical water from the membrane

washing tank can be emptied over 12 nautical miles at a speed of 6 knots. Moreover, it acts as an emergency in case there is wastewater leakage into the bilge. Due to these requirements, the operation of the tank affects the amount of activated sludge retained in the sludge chambers, which affects, among other things, the sludge age and the ability to provide the required oxygen concentration in the aerobic chamber.

Development of the hybrid membrane technology

The introduction of the hybrid membrane technology to passenger ships in international shipping should begin with a way to improve the MBR operation. This will enable water recovery as well as increase energy efficiency and reduce sulphur dioxide emissions to the atmosphere.

While maintaining the flow continuity, it is necessary to consider the problem of excess sludge. A single ship, in line with the operational recommendations, produces excess sludge on a daily basis as part of maintenance. To supply the MBR with excess sludge, we must analyse two options for implementing this solution, considering safety rules, compliance with environmental protection as well as applicable legal regulations. The first option is to include an excess tank to feed the black wastewater retention tank. Due to the specificity of wastewater systems on passenger ships, the black wastewater holding tank, depending on the periods of increased activity on board, often reaches the high-level limit, the reduction of which depends on the efficiency of wastewater treatment by the reactor. Accordingly, it might turn out that emptying the excess sludge tank would not be possible due to the overfilling of the black wastewater tank. Therefore, the possibility of water recycling could prove problematic. A more efficient solution would be to consider the use of excess sludge as a third source of MBR supply. This would allow the ship to maintain the continuous flow to the MBR and to reduce the discharge of excess sludge or even eliminate it altogether. In the case of a low level of gray wastewater in ballast tanks, a permeate tank could be used as another source of external recirculation water supply.

Conclusions

The last element regarding the MBR as a module of hybrid membrane technology is the connection of the reactor with the ballasting system, aimed at improving the gray wastewater treatment process regardless of the geographical location of the ship and the abandonment of the retention tank, replaced with a connection to all ballast tanks. To ensure the best possible operating parameters, including water recovery, reducing energy losses while maintaining compliance with environ-mental protection as well as in the interest of international water management, the UF/reverse osmosis (RO) hybrid membrane technology, based on the existing infrastructure of passenger ships, could be a step forward to water reclamation. The system uses the MBR unit as a pre-treatment, then the permeate is directed through a constant flow fabric filter and low-pressure pump to the RO unit. This solution will reduce the costs of the production of fresh water and could be used in marine power plant systems, as flash evaporators, used for fresh water production, require a specific engine load to increase the activity of the high-temperature water cooling jacket for effective desalination, which means more fuel burned to achieve the set point and also to reduce the cost of fresh water bunkers in ports. The efficiency of the process will not cause disturbances because the MBR and RO are designed

for constant operation, and any stand-by periods can potentially increase membranes fouling. Therefore, it is necessary to ensure continuity of the flow.

Using the UF/RO hybrid membrane technology in shipbuilding will significantly reduce the environmental footprint and preserve the biodiversity of the seas and oceans while complying with international environmental law. The system will allow the maintenance of sustainable environmental development and set a new model of water management, focused mainly on water recovery. A similar system currently operates in Dubai's Burj Khalifa. The reclaimed water from the Dubai Mall and the Burj Khalifa supply the Dubai Fountain to make up the water lost to evaporation.

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Application of polymerase chain reaction-restriction fragment length polymorphism (RFLP-PCR) in the analysis of single nucleotide polymorphisms (SNPs)

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ABSTRACT

Polymerase chain reaction-restriction fragment length polymorphism (RFLP-PCR) is a technique used to identify single nucleotide polymorphisms (SNPs) based on the recognition of restriction sites by restriction enzymes. RFLP-PCR is an easy-to-perform and inexpensive tool for initial analysis of SNPs potentially associated with some monogenic diseases, as well as in genotyping, genetic mapping, lineage screening, forensics and ancient DNA analysis. The RFLP-PCR method employs four steps: (1) isolation of genetic material and PCR; (2) restriction digestion of amplicons; (3) electrophoresis of digested fragments; and (4) visualisation. Despite its obsolescence and the presence of high-throughput DNA analysis techniques, it is still applied in the analysis of SNPs associated with disease entities and in the analysis of genetic variation of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). RFLP-PCR is a low-cost and low-throughput research method allowing for the analysis of SNPs in the absence of specialised equipment, and it is useful when there is a limited budget.

KEYWORDS: nucleotide polymorphisms, DNA analysis, polymerase chain reaction

Description of the polymerase chain reaction-restriction fragment length polymorphism technique and exemplary applications

Polymerase chain reaction-restriction fragment length polymorphism (RFLP-PCR) is a technique used to identify single nucleotide polymorphisms (SNPs) based on the recognition of restriction sites by restriction enzymes (Saiki *et al*. 1985). These enzymes are used to digest specific fragments of DNA, which are then separated electrophoretically on an agarose or polyacrylamide gel and visualised (Figure 1). RFLP-PCR is an easy-to-perform and inexpensive tool for initial analysis of SNPs potentially associated with some monogenic diseases, such as sickle cell anaemia (Saiki et al. 1985), β thalassaemia (Pramoonjago

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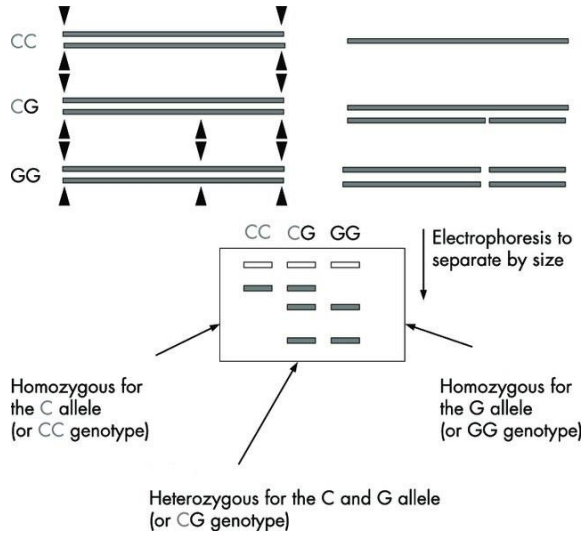

Figure 1. Restriction fragment length polymorphism (RFLP) and detection of alleles. Restriction enzyme digestion of DNA occurs at specific DNA sequences, indicated by the arrow. If a polymorphism (change in DNA sequence) occurs in a restriction enzyme site near a gene of interest, different sized molecules (corresponding to the alleles) will be produced, which are resolved during electrophoresis and subsequently visualised. Adapted with the permission of Harding (2007).

et al. 1999), coeliac disease (Endreffy *et al*. 1992, Catamo *et al*. 2015), phenylketonuria (Meijer *et al*. 1993, Kozák *et al*. 1995) or haemophilia (Křepelová *et al*. 1993, Backfisch *et al*. 1994, Stankovic *et al*. 2005, Herrmann *et al*. 2008, Tasleem Raza *et al*. 2009). RFLP-PCR is also used in genotyping, genetic mapping, pedigree testing and forensics. One of the most interesting applications of this technique is the study of ancient DNA (aDNA) from long extinct organisms, mummified tissues or preserved plant tissues (Hagelberg *et al*. 2015, Orlando *et al*. 2021).

The RFLP-PCR method comprises four steps. In the first step, the genetic material is isolated and the investigated DNA fragment of known length is pre-amplified by using PCR with a pair of specific primers. In the second step, the DNA fragment undergoes restriction digestion carried out by restriction enzymes, which recognise a 4–8 base pair restriction site. In the third step, the digested amplicons are separated via electrophoresis. Depending on the equipment and reagents at hand, the most common type of electrophoresis is slab gel electrophoresis with either agarose or polyacrylamide as the molecular separation matrix (Berg 2012). In the fourth step, the restriction-enzyme-treated amplicons can be visualised by DNA fragment complexation with ethidium bromide (Laber *et al*. 1994) or silver (Budowle *et al*. 1991).

Restriction enzymes are a type of endonuclease that are part of the anti-infection system in bacteria. They differ from one another in the DNA sequences they recognise. Bacteria often possess several restriction enzymes, each specific to a particular short DNA sequence. Type II restriction enzymes are one of the most commonly used in RFLP. These enzymes cleaves the DNA strand at its centre, forming a blunt end, or in a staggered cut, leaving overhangs called sticky ends (Pingoud and Jeltsch 2001). Rebase® is

the most comprehensive repository of restriction enzymes, providing a list of the enzymes, accompanied by information on the restriction enzyme type, the restriction site in a given nucleotide sequence or the presence of isoschizomers. An example list of type II restriction enzymes is shown in Table 1. Chang *et al*. (2006, 2010) developed a database allowing restriction enzyme mining for SNPs in genomes. The SNP-RFLPing analysis provides the SNP contig position, heterozygosity, function, protein residue and amino acid position for coding SNPs (cSNPs) as well as commercial and non-commercial restriction enzymes.

One of the many practical examples of the application of RFLP-PCR in SNP genotyping is that performed by Alavian *et al*. (2018) for the detection of the rs1127354 and rs7270101 polymorphisms associated with the inosine triphosphate pyrophosphatase (ITPA) gene. rs1127354 and rs7270101 SNPs are associated with a functional impairment in ITPase, resulting in anaemia protection in patients with chronic hepatitis C virus (HCV) infection undergoing ribavirin (RBV)-dependent regimens. In a given study, 100 Iranian patients with chronic hepatitis C were genotyped for the detection of rs1127354 and rs7270101 polymorphisms with the help of RFLP-PCR and Sanger sequencing method to validate the results (Figure 2). The results showed that all of the 100 samples tested with PCR-RFLP and sequencing had exactly the same results, with 100% concordance. This demonstrates the utility of RFLP-PCR in studying SNPs with as high efficiency and reliability as Sanger sequencing, but at a reduced cost (Alavian *et al*. 2018).

Despite the presence of high-throughput screening techniques, RFLP-PCR is still applicable for studying the genetic variability of viruses, for

Table 1. Examples of type II restriction enzymes from the Rebase® database. The arrows point to the restriction cut site, creating blunt or sticky ends in the amplicon sequence.

Enzyme	REBASE Number	Source	Recognition sequence	Cut	Isoschizomers
AccBSI	2733	*Acinetobacter calcoaceticus* BS	5′ CCGCTC	5' C C G ▾ C T C 3' 3' G G C G A G 5'	BsrBI, BstD102I, Bst31NI, MbiI
AflII	39	*Anabaena flosaquae*	5′ CTTAAG	5' C T T A A G 3' 3' G A A T T C 5'	BfrI, BspTI, Bst98I, BstAFI, BstPZ740I, Esp4I, MspCI, Vha464I
BamHI	185	*Bacillus amyloliquefaciens* H	5′ GGATCC	5' G G A T C C 3' 3' C C T A G G 5'	AccEBI, AliI, ApaCI, AsiI, Bce751I, Bsp98I, Bsp4009I, BspAAIII,
EcoRI	993	*Escherichia coli* RY13	5′ GAATTC	5' G ▾ A A T T C 3' 3' C T T A A G 5'	Bci528I, Eco82I, Eco228I, FunII, Kpn49kI, Ppu111I
KpnI	1180	*Klebsiella pneumoniae* OK8	5′ GGTACC	5' G ▾ G A A C C 3' 3' C C T T G G 5'	Acc65I, AhaB8I, Asp718I, SthI
HindIII	1151	*Haemophilus influenzae* Rd	5′ AAGCTT	5' A ▾ A G C T T 3' 3' T T C G A A 5'	Asp52I, Asp3065I, BspLAIII, Cfr32I, HinJCII, LlaCI,

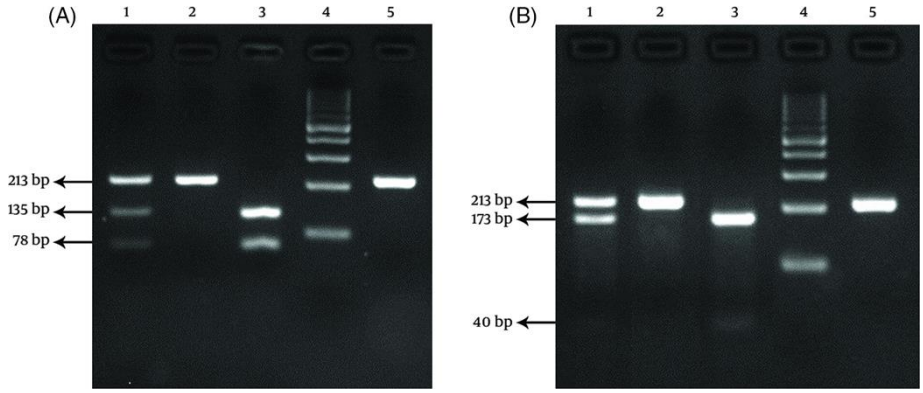

Figure 2. The result of polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) products after digestion by the XceI and MboII enzymes. (A) Gel electrophoresis results of PCR-RFLP products after digestion for rs1127354 by the XceI enzyme. Lanes 1, 2 and 3 were genotyped as CA, CC, and AA, respectively. Lane 4 indicates the 100-bp gene ruler. Lane 5 is non-digested PCR product. (B) Gel electrophoresis results of PCR-RFLP products after digestion for rs7270101 by the MboII enzyme. Lanes 1, 2, and 3 were genotyped as AC, AA, and CC, respectively. Lane 4 indicates the 100-bp molecular gene ruler. Lane 5 is non-digested PCR product. Reprinted with permission from Alavian *et al*. (2018).

example, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the cause of the COVID-19 pandemic. The D614G mutation is characterised by an amino acid substitution of aspartic acid for glycine at position 614 of the spike glycoprotein (S) amino acid sequence. This leads to increased binding to the human cell-surface receptor angiotensin-converting enzyme 2 (ACE2), resulting in increased viral replication in upper respiratory tract cells, thus increasing viral transmis-sibility (Plante *et al*. 2020). The D614G variant is currently the most prevalent SARS-CoV-2 variant in the world (Zhou *et al*. 2021).

Hashemi *et al*. (2020) developed an RFLP-PCR protocol to detect the D614G mutation in SARS-CoV-2 using bioinformatics and software tools. DNA samples from 144 SARS-CoV-2-positive patients were evaluated for the presence of the D614G mutation. In the first step, the spike (S) glycoprotein sequence of SARS-CoV-2 was used to find a compatible restriction endonuclease and primer design. The S-D type is characterised by the presence of a T nucleotide at position 1845, which encodes aspartic acid at position 614 of the amino acid chain. If a T to G mutation has occurred at this position, aspartic acid is replaced by glycine at position 614 of the amino acid chain, which is referred to as the S-G type. The size of the PCR product is 590 base pairs (bp). The enzymatic digestion produces two fragments of 433 bp and 157 bp in length if the T nucleotide is at position 1845. If nucleotide G is at this position, the digestion has no effect on the PCR product, and after agarose gel electrophoresis, one 590 bp fragment is visible. Out of 144 samples, 127 (88.2%) samples belonged to type S-D, 13 (9%) samples were S-G and 4 (2.8%) samples had mixed bands related to both the S-D and S-G types. The results of the given study were consistent with the findings of other investigations examining the frequency of D614G mutations (Bhattacharyya *et al.* 2020). The results of the given study demonstrated the efficiency and effectiveness of the RFLP-PCR

technique in studying the frequency of D614G mutations.

Concluding remarks

The RFLP-PCR technique, despite its obsolescence, is still applicable for studying the frequency of SNPs, especially when there is a severely limited budget and a lack of specialised laboratory equipment.

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Effect of the solvent on the extraction of polyphenols from distillery stillage and on their antioxidant activity

BIOOPEN 2021 – POST-CONFERENCE ARTICLE

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ABSTRACT

The increase in the costs of storage and disposal of post-production residues has resulted in the search for new directions for their recycling, which is closely related to the necessity of protecting the natural environment and promoting a circular economy. Moreover, the apparent interest shown by the food market in raw materials with high antioxidant activity implies an increasing use of by-products. The objective of the study was to determine the effect of the type and concentration of the solvent on the efficiency of extracting polyphenols from distillery stillage as well as their antioxidant activity by using several solvents: methanol:water (70:30 v/v), methanol:water (100:0 v/v), ethanol:water (70:30 v/v) or ethanol:water (100:0 v/v). The DPPH radical method was used to determine the antioxidant activity of the obtained extracts. The normalised variable (NV) and statistical measure (MS) were determined, based on which the effectiveness of the solvents was evaluated. The highest polyphenolic content and the antioxidant activity were obtained by using ethanol:water (70:30 v/v) as a solvent in the extraction of polyphenolic compounds from distillery stillage.

KEYWORDS: DPPH assay, Folin-Ciocalteu, phenolic content, methanolic extract, ethanolic extract

Introduction

One of the main trends in line with the principle of a bioeconomy is sustainable development, which bases its activities on the maximum use of resources of biological origin to protect the natural environment and reduce production costs (Stegmann *et al.* 2020). Following the principle of 'how to get more using less' is to manage and to valorise raw materials and by-products from variable production processes (Okonko *et al.* 2009). Bio-based raw materials with a high recycling potential include, for example, molasses obtained during sugar production (Fan *et al.* 2018), cereal bran (Belc *et al.* 2019) or

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fruit and vegetable pomace (Lin *et al*. 2013, Coman *et al*. 2019).

Currently, special attention is paid to the quality and nutritional value of food. Many products have been shown to have increased susceptibility to oxidative processes that negatively affect their safety. To limit these changes, natural or synthetic substances with antioxidant properties are used (Lourenço *et al*. 2019). A trend has been noticed in which consumers seek to use natural antioxidants. Therefore, the search for inexpensive, efficient and available sources of compounds with antioxidant properties, mainly polyphenols, has become the subject of interest of researchers.

Distillery stillage is an example of a raw material that fits with the current above-mentioned trends. A stillage is a by-product of alcoholic fermentation. It is a valuable source of polysaccharides and volatile fatty acids, as well as natural antioxidants, namely polyphenols. However, there is still no literature data on the use of distillery stillage as a source of bioactive compounds. The stillage composition depends mainly on the processing conditions and the type and quality of the substrates, which means that it may differ among distilleries (Mohana *et al*. 2009). The most commonly used substrates for the production of alcohol are cereals, potato starch and sugar beet molasses (Smuga-Kogut 2015). Therefore, more attention should be paid to the recovery of polyphenolic compounds from by-products of the distillery industry.

The production of alcohol is growing every year because this raw material is used in the chemical, pharmaceutical, cosmetic and food industries, among others (Kharayat 2012). The United States and Brazil produce 94 billion litres of ethanol annually, which is approximately 85% of the global alcohol production (Kharayat 2012). The European Union has implemented a programme obliging the use of biofuels in transport fuels (14.0% by 2025 and 19.7% by 2030) (Krzywonos *et al*. 2015). Alcohol plays a key role in the development of the global economy, but it is also a source of environmental pollution; indeed, 1 l of the produced spirit yields 9–14 l of the by-products. Distillery stillage is characterised by a high content of biodegradable organic matter (chemical oxygen demand [COD] from 15 to 176 g O_2/l) (Melamane *et al*. 2007). The stillage causes a serious ecological problem due to the high concentration of nitrogenous compounds, low pH, high temperature and dark brown colour resulting from the presence of poorly biodegradable melanoidins (Fito *et al*. 2019). Therefore, it is crucial to dispose of the stillage. Until now, the main direction in the management of the by-products of the distillery industry has been their use as fertilisers (Satyawali and Balakrishnan 2008), feed ingredients (Djukić-Vuković *et al*. 2015) and biofuel production substrates (Caruso *et al*. 2019). However, the by-products of the agri-food industry have strong antioxidant properties. Therefore, scientists are increasingly involved in research on bioactive compounds due to their important role in the prevention and treatment of the most serious diseases of civilisation, including heart disease, diabetes and cancer. In food production, they can be used as new ingredients in innovative products or as food additives (Laufenberg *et al*. 2003). The actions taken are in line with the trend of searching for natural compounds with antioxidant properties in place of synthetic antioxidants.

Considering the amounts that are generated, the high nutritional value and the potential strong antioxidant activity, it seems beneficial to use distillery by-products as a source of polyphenols or an

additive to enrich the composition of certain food products. There is a lack of data on the characteristics of stillage in terms of the content of polyphenols and their antioxidant properties. Moreover, the nature and polarity of the solvent used for the recovery of these compounds are important in determining the antioxidant properties. These parameters can significantly influence the hydrogen atom transfer (HAT) or single electron (SET) mechanisms, which are crucial for measuring antioxidant properties (Perez-Jimenez and Saura-Calixto 2006). Hence, the critical point of the research seems to be the selection of an appropriate solvent for extracting polyphenols. Therefore, the objective of the study was to determine the effect of the type and concentration of the solvent on the efficiency of the extraction of polyphenols from distillery stillage and on their antioxidant activity.

Materials and methods

Materials

In this study, distillery stillage from the production of concentrated, crude ethyl alcohol from cereals (a company in north-eastern Poland) was used.

Extraction of polyphenols

The following solvents were used for the extraction: ethanol:water (100:0 v/v) (E 100%), methanol:water (100:0 v/v) (M 100%), ethanol:water (70:30 v/v) (E 70%) and methanol:water (70:30 v/v) (M 70%). To 1 g of the freeze-dried distillery stillage, 10 ml of the solvent was added. The mixtures were shaken for 45 min at room temperature (25 °C) protected light. The extraction process was carried out in triplicate.

Total polyphenolic content

The concentration of total polyphenolic compounds was determined using the colourimetric method with the Folin-Ciocalteu reagent (Singleton *et al*. 1999). This measurement is based on the reversible reduction of molybdenum(VI) present in the Folin-Ciocalteu reagent to molybdenum(V) by phenols in an alkaline medium. For the reaction, 0.25 ml of extract was mixed with 0.125 ml of Folin-Ciocalteu reagent; next, 0.5 ml of a 14% Na_2CO_3 solution was added to the flask. Distilled water was then added to adjust the volume to 10 ml. After 30 min of incubation, the absorbance was measured at 760 nm and compared with the control sample, which was prepared analogously, except using water instead of the extract. A standard curve was prepared using gallic acid (Figure 1), and the polyphenolic content is expressed as gallic acid equivalents (GAE) in mg/ml of sample.

Test with the 2,2-diphenyl-1- picrylhydrazyl (DPPH) radical

The antioxidant activity was measured using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method according to Moure *et al*. (2001). Two hundred microlitres of the extract was added to 2 ml of DPPH solution at a concentration of 0.033518 g/250 ml of methanol. The method is based on determining the degree of reduction of the DPPH radical by antioxidants contained in the sample. The decrease in absorbance was measured at 515 nm for 16 min. The antioxidant activity was calculated according to the formula:

$$\text{Antioxidant activity (\%)} = \frac{(A_0 - A_t)}{A_0} \times 100$$

where A_0 is the initial absorbance of the DPPH solution and A_t is the absorbance of the DPPH solution after 16 min.

Statistical analysis

To determine the efficiency of the solvents used for the extraction of polyphenolic compounds from the distillery

stillage, the unitisation of the variables was used (Guzik *et al*. 2005). Using this method, variables ranging from 0 to 1 were obtained. The STATISTICA 13.1 software (StatSoft) was employed for statistical analysis. The following formula was used for the calculations:

$$NV = (X_J - X^{min}_J)/(X^{max}_J - X^{min}_J),$$

where NV is the normalised variable, X_J is the average value of the variable, X^{max}_J is the maximum value of the variable, X^{min}_J is the minimum value of the variable and J is the type of solvent.

A statistical measure (M_S) was used to determine the ability of solvents to extract effectively bioactive compounds from the distillery stillage. It was calculated according to the following equation:

$$M_S = 1/p \sum N_V,$$

where M_S is the statistical measure, p is the number of variables and N_V is the normalised variable.

Results

The total polyphenolic content was determined by the Folin-Ciocalteu method and is reported as GAE equivalents by reference to the standard curve (Figure 1). Table 1 shows the total polyphenolic content in the distillery stillage and their antioxidant activity. These values depended on the solvent used.

The highest polyphenolic content was in the extracts obtained with the use of E 70% (0.134 ± 0.009 mg GAE/ml) and M 70% (0.121 ± 0.011 mg GAE/ml). The lowest polyphenolic content was obtained when using solvents without the addition of water: 0.064 ± 0.021 mg GAE/ml and 0.078 ± 0.018 mg GAE/ml, respectively, for M 100% and E 100%.

All extracts had strong DPPH radical scavenging activity; the scavenging activity after 16 min of incubation ranged from 64% to 88% of the initial radical concentration (Table 1). Figure 2 shows the DPPH radical scavenging activity over time. Among the solvents used, the

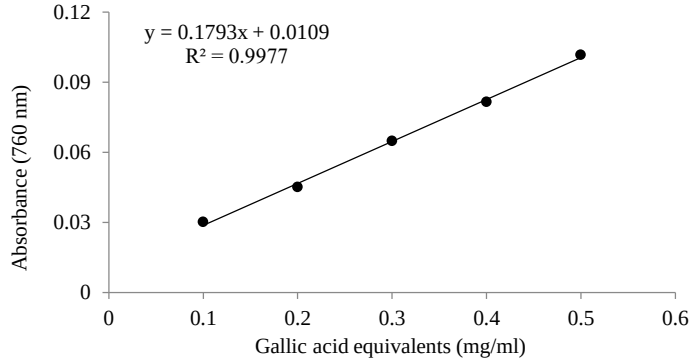

Figure 1. Standard curve for the determination of total polyphenolic compounds in extracts.

Table 1. Total polyphenolic content in distillery stillage and their antioxidant activity.

Solvent	Total polyphenolic content (mg GAE/ml)	Antioxidant activity (%)
E 70%	0.134 ± 0.009	88 ± 0.010
E 100%	0.078 ± 0.018	70 ± 0.021
M 70%	0.121 ± 0.011	78 ± 0.013
M 100%	0.064 ± 0.021	64 ± 0.030

mixtures with water were characterised by higher polarity, and the obtained extracts showed the highest content of extracted polyphenolic compounds and high antioxidant activity. The highest antioxidant activity was in the E 70% (88% ± 0.010%) and M 70% (78% ± 0.013%) extracts. In the case of the M 100% and E 100% extracts, the DPPH radical scavenging activity ranged from 64% ± 0.030% to 70% ± 0.021% after 16 min of incubation.

The statistical measures were calculated using the unitisation of the variables. Table 2 shows the efficiency of the solvents that were used to determine the total polyphenolic content and their antioxidant activity in the stillage extracts.

The E 70% extract had the highest value of the statistical measure (0.90 ± 0.131) and the M 100% extract had the lowest value (0.22 ± 0.282). These data are correlated with the total polyphenolic content and the antioxidant activity obtained in this study. The highest total polyphenolic content and antioxidant activity were obtained by using E 70% as the solvent in the extraction of polyphenolic compounds from distillery stillage.

Discussion

Polyphenolic compounds are secondary metabolites of plants, differing in terms of chemical properties, structures and molecular weights (Wilska-Jeszka 2007). These compounds are generally soluble in water (Shahidi and Naczk 2011). Due to their proven beneficial effects on the human body, they are of interest to medicine, science and food producers (Pandey and Rizvi 2009). The development of an efficient procedure for polyphenolic compound extraction from different sources is a challenge due to the complex matrix, the structural diversity of phenolic compounds and

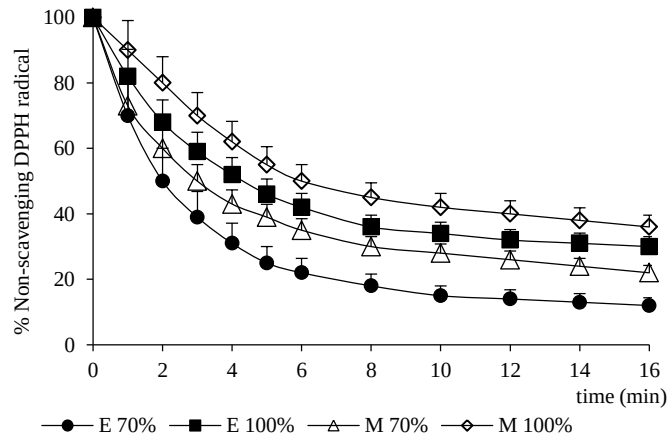

Figure 2. DPPH radical scavenging activity by distillery stillage extracts.

Table 2. Assessment of the effectiveness of the solvents used.

Solvent	N_{V1}	N_{V2}	M_S
E 70%	0.81	0.99	0.90 ± 0.131
E 100%	0.30	0.34	0.33 ± 0.032
M 70%	0.80	0.92	0.86 ± 0.081
M 100%	0.24	0.20	0.22 ± 0.282

their interaction with other cellular components. For this study, distillery stillage (a by-product of crude ethyl alcohol production) was selected as the source of polyphenols. The efficiency of polyphenol extraction mainly depends on the solvent that is used (Azmir *et al*. 2013). Organic solvents (extractants) – such as acetone, ethyl acetate, methanol, ethanol and propanol, or mixtures thereof – are used due to their high selectivity for polar compounds (Araujo *et al*. 2015). From the most commonly used solvents, ethanol is more polar than methanol, extracting preferentially more lipophilic and structurally complex phenolic compounds; moreover, ethanol is less toxic than methanol (Araujo *et al*. 2015). Therefore, this study investigated the polyphenolic content recovered from distillery stillage with two extraction solvents (ethanol, methanol) applied as pure solvents or mixtures with water (E 70% and M 70%).

In the present study, it was confirmed that ethanol was a more effective solvent than methanol in recovering polyphenolic compounds from the distillery stillage. The use of E 70% resulted in a total polyphenolic content of 0.134 mg GAE/ml, while the use of M 70% gave a total polyphenolic content of 0.121 mg GAE/ml. Librán *et al*. (2013) identified the most effective conditions (treatment time and ethanol concentration) for the extraction of phenolic compounds from grape marc and determined the polyphenolic content and the antioxidant activity of the extracts. The highest recovery of phenolic compounds (3.12 mg GAE/g grape pomace) was recorded after a 2 h extraction in a 75% liquid mixture of ethanol. Extending and shortening the extraction time did not increase the extraction efficiency. The highest extraction efficiency was obtained using a 75% ethanol solution because the higher concentration of ethanol led to a reduction in the antioxidant and organoleptic properties of phenolic compounds. Ryznar-Luty *et al*. (2009) noticed that the polyphenolic content in distillery residues depends on many factors, including the quality of the raw material tested as well as the place of origin and the type of raw materials used for alcoholic fermentation. The authors also noted that during transport, distillery residues are exposed to environmental factors such as temperature, humidity and environmental pollution, which also affect their polyphenolic content.

In the case of the analysed extracts obtained from the stillage, solvents of different polarity were used. There were significantly higher polyphenolic content and antioxidant activity in extracts obtained using a mixture of alcohol and water than with 100% solvents. When analysing the influence of the solvents on a group of polyphenolic compounds, there was a correlation between higher solvent polarity and higher antioxidant activity. These results were obtained with different amounts of water in the solvent, a finding that shows variability of the antioxidant potential in different environments. Specifically, hydrophilic antioxidants are more effective in a non-polar system, while lipophilic antioxidants show a better effect in a polar solution (Porter *et al*. 1989). The activity of phenolic acids increases significantly if they contain two ortho hydroxyl groups in the molecule. Such compounds with high antioxidant activity include, for example, ferulic acid, and the presence of the third hydroxyl group causes a further increase in antioxidant activity, as is the case for gallic acid, among other molecules (Rosicka-Kaczmarek 2004).

The strong reduction potential of the stillage extracts was verified in the DPPH radical scavenging assay. The ability to scavenge free radicals depends on the content of substances with antioxidant

properties. The lower polyphenolic content in methanol compared with ethanol extracts was reflected in a reduction in DPPH radical scavenging activity by 10% (mixture of methanol and water) and 6% (pure methanol). Emmons and Peterson (1999) reported that the activity of polyphenolic compounds to reduce the DPPH radical depends on the location and the number of hydroxyl (–OH) and methoxy (–OCH3) groups. Peterson *et al*. (2001) showed that the decrease in polyphenolic content in oat grain causes a decrease in the DPPH radical reduction activity. The authors found a significant correlation between the polyphenolic content and the ability to eliminate the DPPH radical.

The antioxidant activity of phenolic compounds, apart from their primary activity to break free radical reactions, is also based on secondary mechanisms such as the activity to scavenge radicals, to chelate heavy metal ions and to decompose the formed peroxides (Moure *et al*. 2001). Therefore, to characterise fully the raw materials in terms of their antioxidant properties, it is important to use various methods. The tested ethanol extracts from the distillery stillage showed a relatively high content of polyphenolic compounds and their antioxidant activity, determined by the reduction of the DPPH radical.

Conclusions

The extracts from the distillery stillage were characterised by a diverse content of polyphenolic compounds and antioxidant activity resulting from the use of various solvents for extraction. However, the ethanol extracts had a higher polyphenolic content than the methanol extracts. The E 70% extract had the highest polyphenolic content and antioxidant activity. In addition, the polyphenolic content correlated with the antioxidant activity. There was higher polyphenolic content and antioxidant activity in extracts obtained with 70% solvent solutions compared with 100% solvent solutions, a factor that is related to the change in the solvent polarity. The results indicate a potential use of by-products from the distillery industry as a source of natural bioactive compounds. The observed antioxidant activity can be attributed both to the mechanisms exerted by phenolic compounds and to the synergistic effect of various phytocompounds. However, further research in this area should be carried out to determine the profile of phenolic compounds and the spectrum of their activity.

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New trends or return to traditional methods in the production of grain spirits?

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ABSTRACT

This review article is based on scientific and popular science publications as well as articles from branch magazines that refer to the production of alcohol in Poland based on traditional grain raw materials. New trends in the production of broadly understood alcoholic beverages point to a return to traditional raw materials and production methods, preferably carried out in small, traditional distillery – crafted beers, local wines or spirits. Consumers desire a return to tradition, however, it is equally important to maintain the right quality and efficiency of production. The future of alcoholic beverages based on malted and unmalted cereals in Poland is associated with the search for specific varieties of cereals, their mixtures as well as fermentation and distillation processes that will allow the production of 'craft spirits'.

KEYWORDS: rye, barley, cereal malts, alcoholic fermentation, natural products, agricultural distillate, spirit beverages

Introduction

The history of alcoholic beverages manufacturing dates back to the beginnings of humanity – over 10,000 years BC. There was wine made of figs, dates and grapes in China, Egypt, Persia, Greece and Rome. In turn, the first records of beer production in Mesopotamia are from 4000 to 2000 BC. The history of the distillation process, which is the precursor to today's distilled alcoholic beverages, dates back to the 8th century AD, when Arab alchemists invented the process of 'smoking wine'. Mention of the first distillation apparatus (producing *Aqua vitae* or *Aqua ardens*) comes from the 13th century. Significant development and modifications of distillation technique and later purification of the so-called raw alcohol/spirit by using rectification apparatuses took place in the 18th and 19th centuries. At that time, raw material other than fruit started to be used for fermentation – potatoes, molasses and cereals – and there was a rapid development of spirit industry at

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a high-volume scale (Cieślak and Lasik 1979; Rogala 2004).

Polish spirits industry

Spirit beverages produced in Poland are well known and popular all over the world. Our country is the largest vodka producer in the European Union and the fourth largest in the world. Only Russia, Ukraine and the United States have a higher spirit volume produce then Poland. Export of spirits contributes significantly to the improvement of the country's trade balance, and the value of the exported vodka was 142.4 million EUR in 2016 (Związek Pracodawców Polski Przemysł Spirytusowy [ZP PPS] 2021). On average, 71% of exported spirit beverages go to the European Union countries – France is one of the largest importers – and 16% go to the United States. Polish vodkas are well known and recognised around the world.

According to the European Parliament and Council Regulation (EU) (2019/787), vodka is a spirit drink produced from ethyl alcohol of agricultural origin (i.e. rectified spirit) obtained following fermentation with yeast of either potatoes or cereals or both, or other agricultural raw materials, distilled so that the organoleptic characteristics of the raw materials used and by-products formed in fermentation are selectively reduced. This may be followed by additional distillation or treatment with appropriate processing aids, including treatment with activated charcoal, to give it special organoleptic characteristics. The mini-mum alcoholic strength of vodka shall be 37.5% vol. The regulation also set limits for the content of volatile by-products, with particular emphasis on methanol.

Polish Vodka is protected by a geographical indication that proves its quality and reputation due to the region of production. The Act of 18th October 2006 (as amended) on spirit drinks and registration as well as protection of geographical indications for spirit drinks defines the raw material for ethyl alcohol of agricultural origin used in the production of Polish Vodka. Ethyl alcohol produced from rye, wheat, barley, oats, triticale or potatoes cultivated in the territory of the Republic of Poland may be used. Moreover, all stages of production should take place in the territory of the Republic of Poland. Products benefiting from this geographical indication may also be aged to give them special organoleptic properties. The geographical protection of Polish Vodka is to emphasise the tradition and heritage of Poland in the production of spirit beverages, and to draw attention to the traditional raw materials used in the production of vodka.

Cereal grains and malts as raw materials for the production of spirit drinks

Among the starchy raw materials used to produce spirits, the undisputed leader in the production of vodka is rye, which has been used in the production of spirit distillates since the Middle Ages. This vodka, formerly known as okowita (Latin: *aqua vitae*), was described by the Frenchman Sieur d'Hauteville – steward at the court of the Polish king Jan Kazimierz (17th century) – who wrote: 'Vodka is runned in Poland from grain. It is in no way inferior to either the strength or the goodness of wines made with wine yeast (i.e. winemakers)' (Jarociński and Jarosz 1980).

The superiority of rye over the other types of cereals is due to its minimal growth requirements. The low habitat requirements of this species enable it to be cultivated in poorer soils and allow it to survive the unfavourable conditions of winter, as well as periods of water scarcity. The soils in which rye grows do not require a strong support with

fertilisers; this factor is reflected in its quality. Due to a moderate supply of natural nutrients, rye grains are devoid of ingredients that may deteriorate the quality of the ethyl alcohol obtained (Dzienis 2018).

The tradition of making rye vodka has survived to the present day, and rye distillates are valued in the production of super premium vodkas, such as Belvedere Vodka or Wyborowa. Appreciated for their highest quality and unique, characteristic for the raw material used, taste and aroma have found many consumers around the world (ZP PPS 2021).

Changing trends and customer expectations have forced the producers of alcoholic beverages to be more creative in producing unique drinks and moving their production towards eco-friendly products. In their new creations, the producers convert traditional raw materials, such as cereal malts that have been used as enzyme sources, in new roles of flavour, taste and small enhancement components. At the same time, they need to meet the strict quality regulations of different countries, look for process efficiency parameters and understand all fermentation and distillation processes to share the knowledge with the customers, who 'want to understand, not only consume'. For this reason, many scientists have undertaken research on alcoholic products using traditional raw materials, such as cereal grains and malts, to produce the so-called 'crafted vodkas', which are most often made from unrectified/raw spirits. An example of such spirit beverages are drinks called in Polish 'okowita' (category 'spirits/ grain spirits' according to the Regulation EU 2019/787). These spirits are produced exclusively by the distillation of a fermented mash of whole grain cereals and have organoleptic characteristics derived from the raw materials used. The addition of other alcohol or flavours is not allowed (EU Regulation 2019/787).

Cereal grains can be used in the spirit industry in two ways: as a basic raw material (the main source of carbohydrates, namely starch) or as a supportive raw material in the form of malt, as a source of natural amylolytic enzymes (Kaukovirta-Norja *et al*. 2004). These enzymes are represented by α-amylase – catalyses the hydrolysis of starch to dextrins and liquefying the medium, and β-amylase – a saccharifying, maltogenic enzyme. These enzymes are necessary for the hydrolysis of starch during the hydrolysis/mashing to fermentable sugars.

Traditionally, barley malt has been used as a source of enzymes in the production of okowita. The process is still used in the production of traditional whisky/whiskey, while the industrial scale producers more likely use comercial enzymes. Commonly used enzymes are bacterial α-amylases (*Bacillus licheniformis*, *Bacillus stearothermo-philus*) and mould glucoamylases (*Aspergillus niger*, *Aspergillus oryzae*). The use of commercial enzyme preparations has many advantages, guaranteeing high efficiency and stability of industrial processes, but many of their features are a consequence of the use of genetically modified microorganisms (GMOs) for their production (Czupryński and Kotarska, 2011). The GMO approach is contrary to the assumptions of organic production. According to the Council Regulation (EC) 834/2007, the production of products from or using GMOs is prohibited in the production of organic products. The definition of 'ecoproduction' in the context of spirit production naturally guides the thoughts and actions of producers towards traditional production methods using endogenous amylolytic preparations, for example, barley malt or rye malt.

Strąk and Balcerek (2016) studied the effectiveness of saccharification of cereal starch using malts as a source of amylolytic enzymes and the efficiency of alcoholic fermentation. Distillery mashes were prepared from unmalted rye of the Dańkowskie Diament cultivar with 30% (w/w) of wheat, rye and barley malts. For comparison, the unmalted-rye-based mashes were fermented using enzyme preparations of microbial origin. Figure 1 shows that the used malts, with particular emphasis on wheat malt, are suitable for enzymatic hydrolysis of starch. Mash with the addition of malts was characterised by different ethanol biosynthesis efficiencies. The highest efficiency (83.69% of the theoretical), similar to the rye fermentation with a commercial enzyme (86.60%), was observed in the sample with wheat malt. The lowest yield, despite the high enzymatic activity and the highest sugar utilisation, was achieved in mash sample with rye malt (75.74%). The authors explained this phenomenon by the possible presence of compounds inhibiting yeast fermentation activity in the mash. The mash with barley malt achieved results slightly higher than that with rye malt. Rye grain contains a relatively high concentration of non-starch polysaccharides (NSPs) that are composed predominantly of arabinoxylans (pentosans), β-glucans and cellulose. The detrimental influence of soluble NSPs is mainly associated with their viscosity and physiological effects on the digestive medium. Soluble NSPs increase medium viscosity, generally hampering the digestion process, whereas insoluble NSPs impede the access of endogenous enzymes to their substrates by physical entrapment (Hübner *et al.* 2010), which can result in reduced efficiency.

Regarding the chemical composition of the obtained distillates, the use of malts as a source of enzymes resulted in decreased concentrations of undesirable chemical compounds such as methanol and acetaldehyde. This reduction positively influenced the quality of distillates and their organoleptic values (Strąk and Balcerek 2016).

Balcerek *et al.* (2016) determined the efficiency of rye and barley starch hydrolysis in mashing processes using cereal malts as a source of amylolytic enzymes and starch; they also aimed to establish

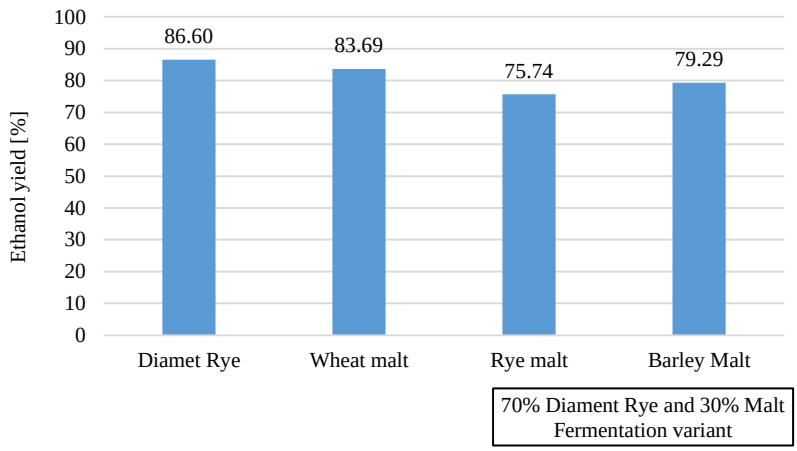

Figure 1. Efficiency of ethanol biosynthesis during the fermentation process of cereal mashes (adapted from Strąk and Balcerek 2016).

the volatile profile of the obtained agricultural distillates. The raw material used were unmalted rye (Dańkowskie Diament variety) and rye and barley malts mixed 50%/50% with unmalted cereals. Two methods of pre-treatment of unmalted cereals were tested, namely pressureless starch liberation (PLS) and thermal-pressure starch liberation. All experiments were performed on a semi-technical scale and then verified under industrial conditions. The results showed that the efficiency of rye fermentation with rye malt in the PLS method was 77.2% (semi-industrial) and 76.2% (industrial test). The sample with barley malt had slightly lower results (76.5% and 75.5%, respectively). Compared with the findings from earlier studies, it can be deduced that increasing the malt content in the mash from 30% to 50% (w/w) did not significantly increase the ethanol production. The authors observed signi-fycant differences in the efficiency of ethanol biosynthesis depending on the starch liberation method. The pressure-thermal starch liberation method contri-buted to higher efficiencies both on the semi-industrial (81.9%–87.0%) and industrial (84.8%–87.1%) scales com-pared with the PLS method (77.2% –80.0% on a semi-industrial scale and 75.5%–77.5% on an industrial scale) for all samples studied. The reasons for higher fermentation efficiency in mashes prepared with use of the pressure-thermal method might be due to the faster release of fermentable sugars during mashing, and their utilisation by yeast (Balcerek *et al*. 2016).

Volatile fermentation by-products and their effect on the quality of spirits

During the mashing, fermentation of grain raw materials and distillation processes, apart from ethyl alcohol, other volatile compounds are synthetised. These compounds include carbonyl com-pounds (e.g. acetaldehyde, isovaleric aldehyde, furfural), carboxylic com-pounds (e.g. acetic acid, propionic acid, valeric acids), esters (which usually occur at the highest concentration of ethyl acetate) and higher alcohols, commonly known as fusel oils (including 1-propanol, 3-methyl-1-butanol, 2-methyl-1-butanol, and phenylethyl alcohol), among others. The level of by-products is usually about 0.5% of the ethyl alcohol content in the distillate (Jarociński and Jarosz 1980). According to the recommendations of the Polish Standard (PN-A-79523:2002), the maximum concentration of higher alcohols in agricultural distillate used for Starka production is 5 g/l absolute alcohol. Traditionally, some types of distillates are used without further puri-fication for alcoholic beverages pro-duction. For example, agricultural rye distillate is used for the production of Starka, rum is obtained from sugar cane molasses and whisk(e)y is obtained from barley malt distillates (Jarociński and Jarosz 1980). In these spirits, it is crucial to maintain an appropriate balance between fermentation by-products and ethanol concentration to keep the desired organoleptic properties of the finished product. For this purpose, batches of pro-duced distillates are aged for a specific time in oak barrels and then combined according to their best organoleptic and quality parameters to create a unique spirits (okowita).

The volatile compounds other than ethyl alcohol are undesirable compounds in the pure vodka. Therefore, the raw spirit/agricultural distillate is purified by a rectification process to reduce impur-ities selectively, and then diluted with water to a certain alcoholic strength.

The increased requirements regarding the quality and production of food and alcoholic beverages (especially on or-ganic products) are factors indicating that technological innovations should be

applied both to improve the quality of traditional spirits and to create new, original spirit drinks. An interesting group of agricultural distillates has revealed spirits produced from cereal grains mixed with cereal malts as a source of amylolytic enzymes and starch. Researchers have shown that when malts are used in alcoholic beverage production, they impact the level of fermentation by-products in the distillates (Strąk and Balcerek 2016; Balcerek *et al*. 2016).

The most commonly analysed impurities of spirit distillates are methanol, acetaldehyde and higher alcohols (Biernacka and Wardecki 2012). Methanol is generated through hydrolysis of methylated pectins present in plants and fruit. While methanol does not directly affect the flavour of the distillate, it is subjected to restrictive controls owing to its high toxicity (Adam and Versini 1996). The content of methanol in alcohol of agricultural origin should not exceed 30 g/hl 100% alcohol. However, there are some differences allowed due to the fermentation ingredients used, for example, 10 g/hl for vodka, 1000 g/hl for grape marc spirit and 1500 g/hl for fruit marc spirit (Regulation EU 2019/787). The methanol concentration in the distillates produced from malted and unmalted cereal grains did not exceed 10 g/hl in the agricultural distillate, which is in accordance with the above-mentioned EU regulation for spirit (Strąk and Balcerek 2016). Balcerek *et al*. (2016) presented a slightly higher result for methanol (less than approximately 20 g/hl 100% alcohol), but it was still within the limits for spirits of agricultural origin.

Conclusions

Although the malted ingredients use to produce alcoholic beverages and the technology by which they are converted have been known for a long time, the new approach for the malted cereals as a well-known flavouring component and process supportive ingredient need additional scientific investigation and deeper understanding. Based on our analysis of the literature, we conclude that the appropriate selection of malted and unmalted cereals, appropriate production technology and proper blending with rectified spirits can open the door to the production of a wide range of 'crafted vodkas', in which the most important factor will be the unique composition and organoleptic properties confirmed by the highest quality of this delicious drink. We believe that tradition can go hand-in-hand with innovative approaches to create new trends in alcoholic beverage production.

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Traditional and new raw materials for spirit beverage production

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ABSTRACT

The ethanol production industry is a fast growing branch of the economy in many countries, and there is a rich tradition of spirit beverage production of many unique drinks such as Polish vodka and Starka or Irish and Scotch whisk(e)y, all of which have unique organoleptic features. This variety is possible thanks to different raw materials used for production such as rye, barley or corn and potatoes, as well as technological solutions developed over the generations of manufacturing. Rye deserves a closer look due to its low growth requirements and many different uses as well as its long tradition of cultivation, especially in Poland. On the other hand, manufacturers are currently interested in using new, original raw materials for the production of so-called craft alcohols. Buckwheat is an example of a raw material that can be successfully used in the production of original spirits.

KEYWORDS: cereal grain, pseudo-cereals, starch, fermentation, agricultural distillate, vodka

Introduction

For centuries, the production of spirit drinks has been an important branch of the economy of many countries around the world. In Poland, the production of drinks as vodka and Starka has a long tradition. Countries such as Scotland and Ireland are known for whisk(e)y production, France is known for cognac and calvados, Italy is known for grappa and the United States is known for bourbons, among others. In Asian countries, strong spirit beverages with high ethanol content are also produced. The typical products of this region are drinks such as arak, rum or Japanese whisky, which is based on the production technology of traditional Scotch whisky and has become popular in recent years. These ethanol beverages have unique sensory features that are directly connected to the wide range of raw material used for their production. These features are a direct consequence of intricate, unique procedures included in their production, for example, the use of smoke created from burning peat to dry malt during Scotch whisky production, while in Irish whiskey smokeless hot air is used in this

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process, resulting in more a delicate flavour of the finished beverage (Cieślak and Lasik 1979).

The Polish spirits industry is a crucial part of the economy. Poland is the largest manufacturer of vodka in the European Union and the fourth largest vodka producer in the world, just behind Russia, Ukraine and the United States. Significant volumes of exports contribute to the improvement of the country's trade balance. For several years, the largest importers of Polish spirit drinks have been France and the United States. Other important export destinations are Hungary, Germany, Italy, Bulgaria and Canada. Besides, Polish spirit drinks are sold in such distant markets as Singapore, Hong Kong and Chile. The estimated demand for starchy raw materials for the annual production of ethanol amounts to an average of 420,000 tons of grain and 80,000 tons of potatoes. It should be emphasised that approximately 94% of used raw materials are from Poland.

In Poland, 320 million litres of spirit beverages are produced each year, and up to 57.75 million litres are exported from Poland all around the world. These exports bring a total revenue of 151.3 million EUR. The ethanol production industry in Poland generates 4009 direct jobs and up to 89,000 indirect jobs. In 2013, the excise tax generated 10.1 million PLN (Związek Pracodawców Polski Przemysł Spirytusowy n.d.) in revenue.

Starchy raw material used for the production of spirits

Currently, the most commonly used basic raw materials for spirit beverage production are potatoes and cereal grains such as rye, wheat, and maize. Manufacturers are searching for new cultivars of plant raw materials, with appropriate physicochemical properties, such as starch, protein content (Pietruszka and Szopa 2014) and moisture content as basic indicators of the storability of cereal grains. Indeed, the levels of these factors indicate the risk of mould formation and the growth of other undesirable microorganisms while the material is being stored (Wilkin and Stenning 1989). Moreover, it is important that the processed raw materials allows the manufacturers to obtain desirable organoleptic properties in the finished products, such as smoothness and delicate smell and flavour.

Rye grain – a traditional raw material in the Polish spirits industry

Rye (*Secale* L.), as most cereal crops, belongs to the class of monocotyledons plants (Monocotyledoneae) from the family Poaceae. Rye originally entered Polish territory from central Asia in the 5th century, where it was commonly growing and was treated as a weed on farmlands. This cereal has low soil requirements and can be grown on almost every kind of existing soil type. The rooting system of rye enables it to draw water, essential minerals and substances needed for growth even from layers of soil in which other plants cannot grow. Even without additional supplementation, rye has shown higher yields than other cereals planted on the same field. A high yield is guaranteed by supplementation of the soil with three basic minerals: nitrogen, phosphorus and potassium. Rye also has low heat requirements – thus it can start to grow early in spring – and it is immune to low temperatures (even –25 °C) in harsh winter conditions. However, the yield is determined mostly by weather and the level of agrotechnology involved in the growing process (Ludwicka 2007, Buksa *et al*. 2012).

The main product in rye farming is its grain, which is used in numerous industries, such as baking, milling or

ethanol and spirit beverage production. Rye grain is also used as animal feed, but farmers have gradually lost interest in it because of its low caloric value. Besides the main product (the grain), other parts of the plant can be used in numerous ways. Indeed, straw can be also used as animal feed but also as lignocellulose raw material in the production of second-generation bioethanol, which is becoming more popular every year, for energy production purposes (Kapusta 2016).

Among many different species of rye from *Secale* L., only one (*Secale cereale* L.) is suitable for cultivation; others have not found application in industry. However, within this species there are many cultivars – natural and manmade – that differ in their physicochemical composition and suitability for industrial applications (Jarosz and Jarociński 1980; Górny 2004; Kapusta 2016).

Besides its basic components such as starch (on average 60%), protein (about 12%) and fat (about 2%), rye grain is also rich source of dietary fiber (Figure 1), of which the main part comprises fructans, pentosans and β-glucans, which are soluble in water fractions and have proven pro-health features. Moreover, rye grain also contains phenolic compo-unds (ferulic acid, 3,4-dihydroxycin-namic acid) phytoestrogens and vitamins. Most of these pro-health components are found in the external layers and not in the endosperm (Gąsiorowski 1994; Michniewicz and Gąsiorowski 1994; Michniewicz 1995; Vinkx and Delcour 1996; Nilsson *et al*. 1997; Heinonen *et al*. 2001).

The characteristic features of soil in Poland are light and very light, which makes rye still one of most popular grown cereals in the country; however, in recent decades its growth acreage has decreased systematically. In 2015, GUS (Główny Urząd Statystyczny) reported that rye in Poland comprised 725,000 ha of land, representing 10.7% of all cultivated cereals, while in the 1980s and 1990s, it had been grown on markedly more land. Among rye cultivars grown in Poland are so-called population and hybrid cultivars. The hybrid cultivars differ from the population cultivars by seed material, which in the case of hybrids needs to be specially cross-bred every time. The main difference between the population and hybrid cultivars is their fertility. Researchers have shown that yield of the hybrid cultivar crops are 17%–19% higher than the population cultivar crops. This difference is directly connected to the heterosis effect obtained by cross-breeding male forms and pollinators with a broad genetic basis. This heterosis effect is only valid through

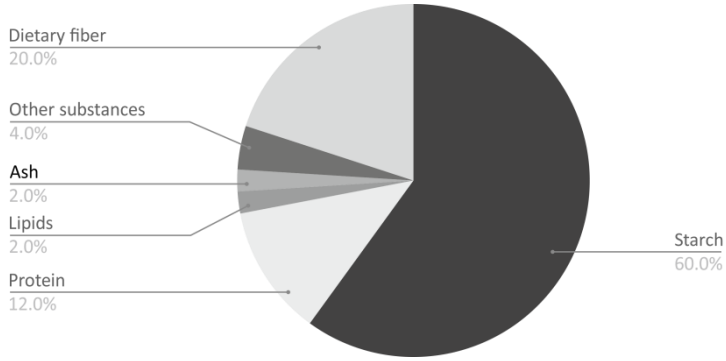

Figure **1.** Average composition of rye grain (adapted from Gąsiorowski 1994).

the first generation (F1); hence, farmers need to purchase the hybrid seed material every year. In the case of the population cultivars, farmers can use the grain grown in the previous year without significant difference in yield height for 2–4 years (Grabiński 2016). The Polish National List (NLI n.d.) currently has 65 registered cultivars of winter rye. Among them are 50 intended for harvesting grain (26 population and 24 hybrid cultivars). Since 2008, the total sugar content of grains from each cultivar has been recorded, information that should be helpful to indicate the cultivars suitable for ethanol production.

Cereal-grain-based spirit beverages

Whisk(e)y

Whisk(e)y is a popular spirit beverage around the world. As defined by Lyns (2003), whisk(e)y is a spirit drink prepared by fermentation of mashes prepared with the use of barley malt and the addition of other cereal grains. The bioconversion process is carried out with use of the yeast *Saccharomyces cerevisiae*. However, there are many types of whisk(e)y produced outside the United Kingdom, from where most popular whiskies (Scotch and Irish whiskey) have originated, but also similar drinks are produced in the United States and even in Japan, where manufacturers produce whiskies with pleasant taste and aroma, such as Scotch whisky.

Scotch whisky

Scotch whisky is a spirit drink produced with the use of barley malt and often with the addition of other cereal grains, mostly wheat and corn. This addition can even reach up to 90% of the total mash mass. Current, wheat is the most used additional raw material. Traditional Scotch whisky is produced in small distilleries all around Scotland, and a number of rigorous requirements need to be fulfilled so that the beverage can be called a 'Scotch whisky'. First, all stages of the production process need to be carried out on Scottish territory from mashes prepared with use of barley malt, the presence of which is crucial. Sweet mashes need to be prepared with only the use of enzymes from malt and fermented only with yeast. Scotch whisky is matured in oak barrels not larger than 700 litres for at least 3 years, but it is commonly aged for at least 8 years. The ageing process also needs to be carried out in Scotland. The finished products should feature the taste and smell of the used raw material and cannot include any other additives than water and caramel (Dunnett 1953; Daiches 1969; Brander 1975).

Irish whiskey

In comparison to Scotch whisky, Irish whiskey has a less of a smoky taste, and more of a rich taste and flavour. A law from the 1980s states that the name 'Irish whiskey' can only apply to beverages that originate from Northern Ireland, obtained after fermentation of mashes prepared with malts and cereals, in contrast to Scotch whisky, the production of which allows the use of an enzymatic preparation. Irish whiskey is also triple distilled to obtain a much smoother and delicate taste, and malt used for the production is dried with use of smokeless hot air. Irish whiskey also needs to be maturated in wooden barrels, not necessarily oak, for at least 3 years (Court and Bowers 1970; McGuire 1973).

American spirit beverages

Spirit drink production – bourbons, rye and wheat-based whiskey as well as Tennessee whiskey – in the United States began in the 18th century. American whiskey is produced similarly to beverages created on the British Isles, however, like in the case of Irish and

Scotch whisk(e)y, the American beverages have some unique sensory qualities due to differences in raw materials and technology. Bourbon is produced with the use of corn as a raw material; the corn grain share should be between 51% and 79% of the overall mash composition. After fermentation, the mash is distilled to not higher than 80% (v/v) alcohol content. The obtained distillate is matured in burned oak barrels; during this process, alcohol content cannot be higher than 62.5% (v/v). Rye and wheat whiskey are produced in a similar fashion: the rye and wheat grains, respectively, need to comprise at least 51%. Tennessee whiskey falls under the same regulation, but to receive the name 'Tennessee whiskey', the production and maturation need to be conducted in the state of Tennessee, and the wood from which barrels for maturation are made also needs to be from Tennessee (Ralph 2003).

Polish vodka

The production of spirit beverages in the Polish territory had been recorded as early as the 14th and 15th centuries. Currently, vodka is defined as a spirit drink created out of ethyl alcohol of agricultural origin, that is, rectified spirits obtained from agricultural distillate, which is produced via fermentation and distillation process from mashes prepared with use of potatoes or cereal grains, such as rye, wheat, triticale, barely, oat or corn. 'Polish vodka' is safeguarded with a protected geographical indication (PGI), which identifies products with a quality or reputation linked to the region where it is produced, and the finished product needs to meet certain requirements. The most basic criteria is that Polish vodka cannot have any additives besides water (in case of so-called pure vodkas) and needs to be produced entirely on Polish soil. Raw materials that can be used for the production of Polish vodka are: rye, wheat, triticale, barely, oat and potatoes, all of which must also be grown on Polish soil. Polish vodka can be matured to obtain the required unique organoleptic properties. This definition is formulated in article 38 of the Act on spirit drinks and registration, and protection of geographical indications for spirit drinks (2006).

Starka

Starka is a natural cereal vodka (okowita), the production of which dates back to the 16th century. According to the historians of the traditional production of Starka, distillate with 55% alcohol content was used, it was placed in oak barrels and later buried in sandy ground for 15–20 years to gain its unique organoleptic features. Currently, for Starka production raw rye distillate with a 91.5% (v/v) alcohol content is used, with higher alcohol content up to 2 g/l of 100% (v/v) ethanol. The distillate is matured in small oak barrels (up to 300 litres) for at least 5 years. The Szczecińska Fabryka Wódek 'STARKA' states that currently the oldest barrel still sealed and maturing is dated to be from 1947, while the oldest Starka for purchase is 50 years old (Polish Vodka Association n.d.).

Microflora of the cereal grains

One of main concerns in the ethanol production industry is the presence of accompanying microflora in raw materials, which can cause further contamination of the mash during the fermentation process. Microorganisms can grow on the above-the-ground parts of plants, the so-called phyllosphere, which is a perfect environment for supporting growth of such microbiota, due to the easy access to a food supply and optimal growth conditions. The development of the accompanying microflora is also

supported by a warm and humid climate (Dix and Webster 1995).

The accompanying microflora in cereal can be divided into two groups. The first group, epiphytic microflora, comprises microorganisms growing on the surface of the plant on the field during the growth process. The second group comprises deep microflora, which get inside the grain during growth and thus develop during the storage process (Broda and Grajek 2009).

Epiphytic microflora include both fungi and bacteria. The main bacterial genera are *Bacillus*, *Flavobacterium*, *Pseudomonas* and *Agrobacterium*. Moreover, there can be pathogenic bacteria also such as *Mycobacterium* spp., *Escherichia* coli, *Clostridium botulinum*, and *Listeria monocytogenes* (Bakken 1997, Maciorowski *et al*. 2007). The fungal genera include moulds such as *Alternaria*, *Cladosporium* and *Rhizopus*, which are safe, but also species generally considered as dangerous for humans and animals, such as *Fusarium* spp. and *Helminthosporium* spp. (Barney *et al*. 1995, Bakken 1997). The moulds belonging to the second group are especially undesirable in the ethanol production industry because they produce mycotoxins, such as zearalenone; deoxynivalenol; and fumonisin B1, B2 and B3. Because mycotoxins are resistant to degradation in the conditions used during ethanol production, mycotoxins are present in the waste material/distil-lery stillage, which is used as animal feed. Moreover, mycotoxins in mashes have a direct impact on the acetaldehyde content in the finished agricultural distillate (Kłosowski *et al*. 2011).

The deep microflora that develops during storage of the grain mostly comprises fungi: the quantity of bacteria during the storage process falls below 1000 cells/g of grain (Broda and Grajek 2009). The fungal genera include *Chaetomium*, *Hansenula*, *Aspergillus*, *Candida* and *Penicillium* (Maciorowski *et al*. 2007).

Buckwheat – a new raw material for agricultural distillate production

Buckwheat has gained popularity around the world due to its low soil requirements and the comparable starch content relative to widely used cereal grains; these factors make it a potential new raw material for spirit beverage production. In some countries, buckwheat is used for the production of alcoholic beverages: French and U.S. distillers use this raw material in whisky production, while in Japan buckwheat grain is processed to make an alcoholic beverage called soba shōchū (Haros and Sanz-Penella 2017).

Buckwheat does not belong to the grass family (Poaceae), as cereals do, and it is therefore referred to as a pseudocereal. There are fifteen species of buckwheat, nine of which are used in agriculture, but only two are grown for food purposes: *Fagopyrum esculentum* and *Fagopyrum tataricum* (Dziadek *et al*. 2016). Buckwheat has a similar chemical composition to cereals (Figure 2). The average starch content in buckwheat grain is about 50%. The content of other macromolecules is 12% proteins, 4% lipids, 2% soluble saccharides, 7% dietary fiber, 2% ash and 18% other substances (Im *et al*. 2003).

Buckwheat is often used in the manufacture of products intended for consumers with coeliac disease. A buckwheat malt is used in the production of gluten-free beer (Deželak *et al*. 2014). In the case of distilled alcohols, the absence of gluten proteins in buckwheat grains is of little interest because all spirits are gluten-free. The distillation process makes it unlikely that allergenic

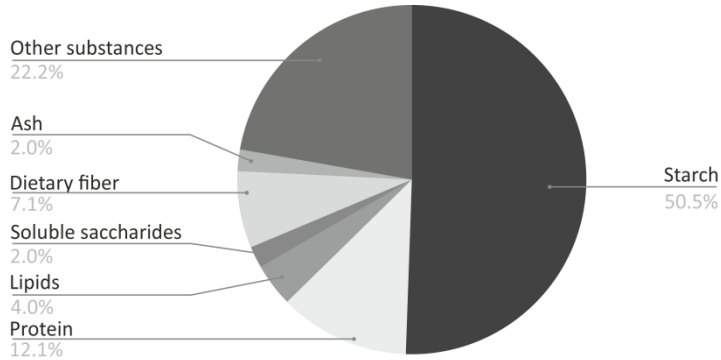

Figure 2. Average chemical composition of buckwheat (adapted from Im *et al.* 2003).

proteins will be carried over into the distillate during this process (European Food Safety Authority 2004). Many researchers have reported on the use of buckwheat for the production of various food products, including alcoholic bever-ages (Starowicz *et al*. 2018). Moreover, the direct interest of Polish producers of spirit drinks to produce new, original beverages prompted us to undertake research on the use of buckwheat grain for the production of spirit drinks. In our study (Ługowoj *et al*. 2020), we assessed the suitability of two cultivars of buckwheat grains, Panda and Kora, for agricultural distillate production. Both cultivars had a similar starch content. Fermentation of the Kora-cultivar-based mashes resulted in a higher fermentation efficiency (up to approximately 85% of the theoretical yield) compared with the Panda-cultivar-based mashes (up to approximately 75%). The obtained distillates contained relatively low concentrations of unde-sirable compounds, such as acetaldehyde and methanol, and revealed pleasant organoleptic features. The results of this study confirm the possibility of using this pseudo-cereal in the production of original distillates with a specific aroma, flavour and raw material identity.

Conclusions

The ethanol and spirits production industry is an important branch of the economy in many developed countries, including Poland. This is due to the fact that ethanol is used in many industries, including chemical, pharmaceutical, food and beverage production, among others. Hence, research in this field is crucial to provide further development. Moreover, there is increasing interest in niche craft products from both micro-distilleries and large spirit plants, which are considering the use of new raw materials while taking into account social attachments and tradition.

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The structure of *Prunus avium* L. crops and their importance for pollinating insects in seed orchards in Poland

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ABSTRACT

The functioning of forests in Poland is often associated with a productive role and the production of wood-based raw materials, disregarding the need to protect forest environments. In practice, the approach to the protection of nature and animals, including insects in forest areas, has changed in recent decades. Many researchers still point to the need to protect the processes taking place in forest environments. Actions are being taken to reduce monocultures in forests and to increase the biodiversity of plants and animals living among crops. A good example described in this paper is the relationship between seed plantations and insects. These relationships may have a positive effect on the fruiting process and seed production in selected tree species. This paper presents an example of the relationship between wild bees and *Prunus avium* L. seed plantations as an example of a positive relationship in which humans as well as pollinating insects can benefit. The structure and size of *Prunus* cultivation in Poland are described and the hitherto harvest of seeds is analysed. The elements of the biology of the *Prunus* species, important for the process of pollination of flowers by insects are also indicated. The study also indicates ways to protect bees in the forest environment.

KEYWORDS: seed production, forest crops, tree yielding, plant cultivation

Introduction

Anthropogenic activities can alter forest environments (Grodzki 2020). A way to counteract this process is to create a forest seed base of various types of trees, conifers and deciduous trees, from which seeds are obtained for further sowing and for the formation of forests (Kocięcki 1965; Fonder 2006; Matras and Fonder 2006). Many authors have pointed to the need for a more multifunctional and sustainable use of forest resources and the rich diversity of species living in forest areas (Fonder 1992; Matras 1992; Matras 2000; Chałupka *et al*. 2011; Szyp-Borowska *et al*. 2012). The overarching goals

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include the need to protect nature and maintain a seed base, allowing for forest districts to meet self-sufficiency in terms of the production of tree seedlings (Matras and Fonder 2006; Kowalczyk *et al*. 2011). Contemporary silviculture focuses primarily on maintaining the high diversity of native species of forest trees (Chałupka *et al*. 2011; Kowalczyk *et al*. 2011). Crops provide, above all, high-quality seed material with known genetic characteristics, which helps to protect the genetic resources of trees (Kowalczyk *et al*. 2011). There are many examples of the need to protect nature in an interdisciplinary manner, but a good illustration of this concept is the relationship between pollinating insects and trees that produce nectar and bee pollen, resulting in the formation of fully developed seeds capable of germination (Baddeley and Watson 2005; Cetinbas and Koyunuu 2006; Szczygieł and Wojda 2008; Chałupka *et al*. 2011, Kowalczyk *et al*. 2011). This process should be supported by active protection of bees in forest areas by introducing various species of trees, shrubs and herbaceous plants to forest monocultures, which will be able to provide food to pollinating insects throughout the year, not only in spring or summer (Kowalczyk *et al*. 2011). It is a necessary element that influences the reproduction of insects and their existence in forest areas and beyond (Wilkaniec and Wyrwa 1994; Frączek *et al*. 2020; Grodzki 2020).

Prunus avium L. in forestry

Forest seed plantations, especially trees that produce nectar and pollen consumed by animals, are a good example of a food base for pollinating insects and bees. An example of a cultivated plant is *Prunus avium* L. (bird cherry), which blooms profusely in April-May and produces nectar and pollen that is valuable for bees (IBG 2021). There are eight such plantations in Poland, comprising 34.78 ha (Table 1). Although their formation has only taken place in the last two decades, they already provide seeds with valuable features in the process of silviculture. So far, the sum of seeds collected from all these plantations has amounted to 991.14 kg, which averages to 28.50 kg/ha (Table 1). These data comes of Krajowy Rejestr Leśnego Materiału Podstawowego in Baza Nasiennictwa Leśnego. Currently, it is the base of crops where there are problems with keeping trees in optimal health conditions, which contributes to their fruiting (Ballistreri *et al*. 2013). However, the maintenance of plants and breeding of various strains of these trees is a response to the needs of foresters in the field of forest seed production and the need to use *P. avium* seeds in the process of creating new forest stands, which should not contain a single species (monoculture), but rather constitute a collection of different tree species, providing animals with shelter, food and space to live. The yield of this species depends on the weather conditions during its flowering, which occurs in April and May, and the health condition of the trees. Bird cherry grows wild in Europe, Western Siberia and Central Asia. In Poland, it is found in deciduous and mixed forests. It is a light-requiring species with high soil requirements, preferring dry areas. Trees generally begin to yield at the age of 6–8 years. Abundant fruiting is usually observed at the age of 15 years. The most common form of harvesting ripe fruit is hand picking. The indicators to assess maturity are colour and consistency. Mechanical shakers are also used in seed plantations. Table 1 provides basic information on the structure of *P. avium* crops in Poland, together with an indication of their seed productivity.

Table 1. The basic characteristics of *Prunus avium* L. seed orchards in Poland based on KRLMP (BNL 2019)

Regional forest district	Forest district	Area (ha)	Year established	Total seed harvest until 2020 (kg)	Average seed harvest (kg)
Krosno	Dynów	5.33	2005	70.00	35.00
Gdańsk	Elbląg	4.20	2015	113.65	28.41
Katowice	Kędzierzyn	5.20	2009	19.10	9.55
Lublin	Krasnystaw	6.51	2009	0	0
Poznań	Łopuchówko	2.18	2007	0	0
Katowice	Rudy Raciborskie	2.39	2008	144.39	48.13
Lublin	Świdnik	4.99	2003	575.00	95.83
Szczecinek	Świerczyna	3.98	2010	69.00	34.50

The importance of forest seed crops and forest vegetation for pollinating insects

The vast majority of forest areas are coniferous species with low nutritional value for pollinating insects, including *Apis mellifera* L. honey bees and various species of wild bees (Frączek *et al*. 2020). In the area of forest seed plantations in Poland, conifers (e.g. *Pinus sylvestris* L., *Picea abies* L., Larix Mill.) and deciduous species (e.g. *Tilia cordata* Mill., *Quercus* spp., *Prunus avium* L.), whose main task is the production of seeds for the further creation of forest stands of known, primarily genetic origin (Chałupka *et al*. 2011; Kowalczyk *et al*. 2011). Their role, however, can be perceived much more broadly, as some of them are a crucial source of food, providing pollinating insects mainly with bee pollen and sometimes nectar (Czekońska 2020b; Frączek *et al*. 2020). These crops can also be made available to beekeepers – especially forest seed orchards of *T. cordata* – and they are very valuable to honeybees and other pollinating insects (Czekońska 2020a). Their flowering provides nectar as well as pollen, from which linden honey can be produced (Czekońska 2020b). Other valuable crops for bees are oak plantations, from which bee pollen can be obtained by insects (Frączek *et al*. 2020). Another species is *P. avium*, which also provides valuable food products for bees, here also mainly bee pollen (IBG 2021). In addition, forest areas around seed plantations may include in the forest composition species of trees and shrubs that are valuable for bees and pollinating insects, including: *Padus avium*, *Sorbus aucuparia*, *Acer pseudoplatanus*, *Acer campestre*, *Acer platanoides*, *Tilia platyphyllos*, *Cerasus avium*, Salix spp., *Frangula alnus*, *Berberis vulgaris*, *Viburnum opulus*, *Corylus avellana*, *Ribes nigrum*, *Prunus spinosa*, *Crataegus* spp. and others (Frączek *et al*. 2020).

Protection of pollinating insects in forest areas

When thinking about the protection of insects, one should distinguish between active and passive activities. To increase the population of pollinating insects – and above all, bee species – care should be taken to increase the number of nectar- and pollen-bearing species that are components of forests (Frączek *et al*. 2020). This necessity results from the low abundance of tree and shrub species in forest environments that provide food for insects in the long term (Szwagrzyk *et al*. 2020), which is necessary for the proper existence and reproduction of some species that also live in agricultural

and fruit-growing environments (Wilkaniec and Wyrwa 1994). Forest environments are constantly changing due to human activity, a factor that may limit the development of animals, including invertebrates and insects (Grodzki 2020). The technological processes of tillage have changed significantly. Moreover, various plant-protection products have been used and the number of weeds present in the crop structure has been reduced (Grodzki 2020; Szwagrzyk *et al*. 2020). The need to protect insects in forests has been discussed in more detail so far in Instrukcja Ochrony Lasu (IOL 2012), where it is clearly stated that it is necessary to increase the biodiversity of plant species found in forest areas to increase the biodiversity of pollinating insects.

Conclusions

Increasing the biodiversity of pollinating insects, especially wild bees living in the forest structure, seems to be crucial to preserve the species diversity of these animals as well as to increase the yield of selected tree and shrub species grown in forest seed orchards. An example of such interdependence in a forest environment is the relationship between *P. avium* plantations and the bees from which seeds are produced, which are the basis for new plants. Many more examples of such connections can be found, especially for trees such as *T. cordata*, *Quercus petraea* Liebl. and other species for which the presence of bees and their work is essential in the fruiting of trees. Oak species are also highly wind pollinated and yet are additionally favoured by the work of bees. It can be clearly concluded that the natural environment needs to balance economic goals and animal protection, which may result in more positive changes, which will also benefit humans.

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Towards a better understanding of the bacterial pan-genome

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ABSTRACT

The bacterial pan-genome is a relatively new concept that refers to the number of genes observed in a given set of bacterial genome sequences, either at the intra- or inter-species level. Determining the pan-genome of a given species of bacteria using a large number of strains allows one to compare multiple genes and to determine evolutionary links between isolates. This information can help to determine population structure, diversity in terms of prevalence in a given environment and pathogenicity of microorganisms. Within this review, we explain the most important issues related to pan-genome studies. We also include a brief description of some selected bacterial pan-genomes. Finally, we propose an easy-to-perform workflow to study bacterial pan-genomes that will facilitate non-experts in a pan-genome-based investigation.

KEYWORDS: pan-genome, bacterial pan-genome, genome comparison, Roary workflow

Introduction

A bacterial pan-genome can be defined as the total number of genes observed in a certain group of microorganisms. The pan-genome of individual bacterial species is most often analysed, but some studies focus on broader groups of microorganisms, for example, a genus. The term pan-genome was proposed in 2005 by Tettelin and co-workers (Guimarães *et al*. 2015, Mira *et al*. 2010). Next, Rouli *et al*. (2015) clearly defined a pan-genome, or supragenome, as 'the entire gene repertoire of the study group'. Pan-genome research has become possible due to the development of next-generation sequencing (NGS) technology, which has allowed the sequencing of bacterial genomes (Guimarães *et al*. 2015, Rouli *et al*. 2015).

On the other hand, the idea that the genome of individual strains may differ significantly within a species was born in the 1980s, when using *Escherichia coli* and the technique of electrophoresis in a variable pulse field revealed that the size of the genome of strains of this species was between 4.5 and 5.5 mega base pairs

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(Mbp). A relationship was also observed between the genome size and strain differentiation using the multilocus enzyme electrophoresis (MLEE) method (Mira *et al*. 2010). This method allows bacteria to be differentiated based on the relative migration rate pattern of a large group of intracellular enzymes. The different patterns result from mutations in the genes coding for these enzymes, which change the amino acid sequence of the proteins (Caierão *et al*. 2016).

Types of genes in the pan-genome

The pan-genome is a pool of genes that may occur with different frequency among the studied group of micro-organisms (Costa *et al*. 2020; Guimarães *et al*. 2015). Based on the frequency of their occurrence, genes are assigned to one of three groups. The genes found in the genomes of all the microorganisms analysed are called core genes. The genes found in only some of the genomes studied are termed accessory genes. The third group of genes that make up the pan-genome are unique genes, the presence of which is found only in single genomes (see Figure 1). Depending on the scope of the analysis, unique genes may be strain specific (in the pan-genome analysis of one species) or species specific (when the analysis is carried out at the inter-species level) (Costa *et al*. 2020; Guimarães *et al*. 2015; Mira *et al*. 2010; Rouli *et al*. 2015).

The genes of the aforementioned subtype found during the pan-genome analysis play different roles in the development of microorganisms. It is believed that the core is made of genes responsible for the basic functions of the bacterial cells, including housekeeping, cell division (replication) and homeostasis. Meanwhile, accessory and unique genes play a supporting role in relation to core genes. These genes are related to the growth environment of a bacterial species as well as the virulence of pathogenic bacteria. These genes are acquired through horizontal gene transfer, a phenomenon that can confer an adaptive advantage, and their presence can be a factor that facilitates development relative to strains lacking them (Costa *et al*. 2020; Mira *et al*. 2010; Rouli *et al*. 2015).

Types of pan-genomes

After determining the number of genes in the pan-genome and assigning them to individual subtypes, the next step to characterise it is to determine the ratio of the number of core genes to others. In

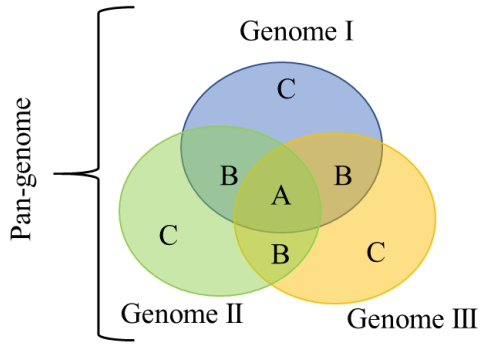

Figure 1. A Venn diagram that represents the three types of genes present in the pan-genome: A – core genes present in all analysed genomes; B – accessory genes, present only in some of the genomes studied; C – unique genes, characterising individual genomes.

addition, the number of new unique genes is observed when adding more genomes to the analysed pool. Based on the results of the second analysis, pan-genomes are divided into open and closed. An open pan-genome refers to when another genome added to the analysed pool increases the number of unique genes. Conversely, when adding more genomes does not increase the pool of unique genes, the pan-genome is termed closed (Rouli *et al*. 2015).

A simple way to determine whether the pan-genome is closed or open is to construct rarefaction curves. This tool is normally used by ecologists to determine graphically when further sampling would not increase the number of newly identified species. Using similar approaches, genes are counted as additional genomic sequences are added to the analysis. The results are presented in a graph of the total number of genes in a pan-genome versus the number of analysed genomes. If the curve reaches a plateau, the pan-genome is termed closed. Open pan-genomes are characterised by the fact that with each genome added, the number of genes increases at a constant rate (see Figure 2) (Mira *et al*. 2010).

Another tool is to apply Heap's law to determine the openness of an analysed pan-genome. Heap's law describes the number of distinct words in a document as a function of the document length. It is represented by the formula $n = k \times N^{-\alpha}$. In a pan-genome studies:

- n is the expected number of genes for a given number of genomes,
- N is the number of genomes and
- k and α are free parameters that are determined empirically.

According to Heap's law, when α is > 1, the pan-genome is considered to be closed, and when α is < 1, the pan-genome is considered to be open (Guimarães *et al*. 2015).

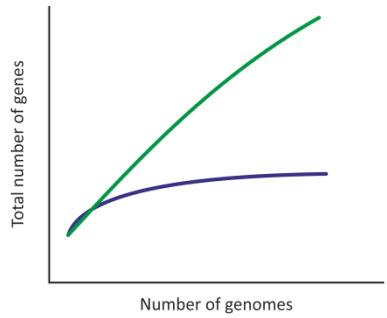

Figure 2. Rarefaction curves for open (green) and closed (blue) pan-genomes.

Examples of pan-genomes of selected bacteria

Within this paper we describe briefly some pan-genome studies of selected gram-negative and gram-positive bacteria. The presented information shows that utilisation of the pan-genome approach in microbiology could extend our understanding of molecular aspects of bacterial diversity, evolution and pathogenesis.

E. coli is an important urinary track pathogen; strain ST131 is becoming a serious problem due to its multi-drug resistance. Decano and Downing (2019) studied a cohort of 4,071 genomes of the ST131 strain to investigate the genetic diversity of the group based on the core and accessory genes. Their analysis indicated that the average number of genes in the pan-genome of ST131 increased as more genomes were added, indicating the open nature of the pan-genome of the entire collection. The authors found 26,479 genes, of which 3,712 genes present in all isolates formed the core. The tested strains formed three clades: A, B and C; these classifications were based on phylogenetic analyses of the ST131 *fimH* gene. Clade C was the largest group. The pan-genome was also tested as an independent sets of genomes. In all sets, the pan-genome was described as open. This comparison revealed inter-

clade but not intra-clade accessory genome divergence, which might result from ecological specialisation of the strains (Decano and Downing 2019).

In another study, the authors employyed inter-species pan-genome analysis to compare the pan-genomes of *E. coli* and *Shigella* spp. to *Salmonella enterica*. This analysis indicated that *Shigella* should not be considered an independent genus – based on pan-genome diversity – because, after examining its genome, it turned out that it did not contain any specific genes not present in *E. coli*. This would mean that there are no barriers in the gene pool between the species. At the same time, *E. coli* and *S. enterica* maintained stable, species-restricted gene pools, despite intensive horizontal gene transfer between the species. Importantly, pan-genome analysis allowed the researchers to complement the current classification of the studied species, providing a new perspective to the understanding of bacterial evolution. Consequently, it can allow researchers to understand the interactions between strains in the environment, to track the evolution of individual lines, to predict the probability of certain diseases after infection with a given microorganism and to improve the treatment process and diagnostic tools (Gordienko *et al*. 2013).

Pseudomonas aeruginosa is the third most frequent opportunistic pathogen found in hospitals. The bacterium is resistant to most classes of antibiotics and causes major infections in immunocompromised patients, including individuals with cystic fibrosis. Understanding evolutionary processes and molecular mechanisms of *P. aeruginosa* on the pan-genome scale could help to explain the ineffectiveness of the designed vaccines against this pathogen and to understand the mechanisms by which *P. aeruginosa* strains avoid the human immune system.

Recently, Freschi *et al*. (2018) used 1,311 strains to update the pan-genome of this opportunistic pathogen. This approach allowed them to define the structure of the population and to determine the number of primary genes and to assign functions to them. Based on their data, the *P. aeruginosa* pan-genome comprises 54,272 genes: 665 core genes, 26,420 flexible genes and 27,187 unique genes. Overall, 33.1% of pan-genomic genes have not been assigned a function, and core genes account for only 1% of the total pan-genome. These findings demonstrated that determining pan-genomes or updating existing ones with larger data sets provides a better understanding of the population structure and evolution of microbes (Freschi *et al*. 2019). In contrast to the above-mentioned study, a previous analysis of 181 *P. aeruginosa* genomes showed that the pan-genome comprises 2,503 core genes (15%), 9,108 additional genes (54%) and 5,209 unique genes and is closed (Mosquera-Rendón *et al*. 2016).

Burkholderia cepacia is a gram-negative bacterium that does not produce spores and cannot ferment glucose. It is common in humid environments (e.g. around plant roots) and is a common cause of opportunistic nosocomial infections, with cystic fibrosis patients being the most vulnerable to infection (Mahenthiralingam *et al*. 2008). Recombination and positive selection are two fundamental evolutionary forces that can be studied by performing comparative genomic analyses. *B. cepacia* species are very difficult to distinguish genotypically and phenotypically due to their high level of recombination, which is strongly supported by about 5.8% of the basic orthologous genes, while 1.1% of these genes support positive selection (Zhou *et al*. 2020). This problem can be solved by using combined methods that ensure proper recognition of species, even those

that are closely related to each other. It is suggested to combine the core-gene-based phylogenetic study with the analysis of digital DNA-DNA hybridization and Average Nucleotide Identity (dDDH/ANI) clusters and the formation of species trees (Jin *et al*. 2020).

The analysis of the genomes of bacteria isolated from cystic fibrosis patients is often a source of valuable information about changes in genomes under the influence of the host's immune system and the therapies used. Phylogenetic analysis of 2,148 ortholo-gous gene clusters from *Burkholderia cenocepacia* isolates collected from 16 cystic fibrosis patients confirmed compliance with patient-specific clades and allowed the observation of pathogen transmission among patients (there was evidence of shared clonal lines), as well as frequent repeated loss of genes and the entire chromosome III (Lee *et al*. 2017). Based on the above-mentioned studies, the analysis of the *Burkholderia* spp. genome has contributed to a more indepth understanding of the phylogenetic tree of these microorganisms, and thus to the development of more effective treatment methods and improved diagnosis of infections.

Staphylococcus aureus often causes hospital- and community-acquired infections that, due to the presence of methicillin-resistant strains, are very difficult to treat and can lead to sepsis and death (Guo *et al*. 2020). Strain antibiotic resistance is one of the major problems of modern medicine; this phenomenon may be better understood by examining the evolutionary pathway and origin of resistance genes in common bacterial pathogens. Indeed, an analysis comparing 152 fully sequenced *S. aureus* strains with 7,529 reference genomes of other bacteria found that 55% of known resistance genes for this bacterium belong to its accessory genome and 27% of them were located in Staphylococcal Cassette Chromosome mec (SCCmec), and in most cases they were acquired laterally from other species (John *et al*. 2019). *S. aureus* co-exists on the skin, throat and nose alongside *Staphylococcus epidermidis*. Approximately half of their genomes are shared, and homologous recombination between the species is rare. However, they contain a significant proportion of interspecific mobile elements, which are genes responsible for metal detoxification, methicillin resistance (SCCmec island) and are associated with the pathogenicity island (SaPIn1) (Méric *et al*. 2015). Genome sequencing allows for the analysis of the structural and evolutionary changes of microorganisms over the years. The analysis of *S. aureus* USA300, which represents a line of methicillin-resistant *S. aureus* strains in the United States, revealed that pan-genome evolved from 2004 to 2010 (Jamrozy *et al*. 2016).

Staphylococcus lugdunensis has unique properties among *Staphylococcus* and occurs on the human skin. This coagulase-negative microorganism can produce various virulence factors and has the ability to cause severe infections, especially in hospital conditions. Interestingly, it is easily treated with antimicrobials, a feature that is quite unheard of for this type of bacteria. Phylogenetic studies of the *S. lugdunensis* genome have shown its high conservation in terms of antibiotic sensitivity and extremely rare methicillin resistance even in hospital conditions, which distinguishes it from all other staphylococci. To investigate the *S. lugdunensis* genome, researchers used 16 different strains from Europe, Asia and North America, isolated between 1988 and 2015. They found *S. lugdunensis* has a very closed pan-genome with a fairly limited number of new genes. This is an infrequent feature for *Staphylococcus* spp., which

have an open pan-genome (Argemi *et al.* 2018).

Bacillus cereus sensu lato is a diverse group of bacteria containing many species found in different environments and exhibiting a variety of phenotypes. Many species of this genus have medical or agricultural significance. The pan-genome of *B. cereus s.l.* consists of approximately 60,000 genes, 598 of which are core genes. The accessory pan-genome consists of 32,324 genes, of which 27,067 are unique. Gene analyses indicate the presence of open pan-genome for *B. cereus s.l.* (Bazinet 2017).

Pan-genome study workflow

With the increased number of studies focused on bacterial pan-genomes, new in silico tools have been developed. Most of them are command-line-based software programs that allow testing the pan-genome-based diversity. The majority of the software programs have been reviewed by Guimarães *et al*. (2015). The major disadvantages of the software programs is that they require computing skills, which might be problematic for users who cannot code. Therefore, within this chapter we propose a simple workflow that allows performing solid pan-genomic and phylogenetic investigation of bacterial species of interest (Figure 3). This workflow should be used as a guide for beginners. It reviews tools and briefly describe their usage, but we advise that one should expand their computational skills.

For the purposes of this workflow, we recommend installing the latest version of Ubuntu, which is a Linux program based on Debian recommended for beginners (Möller *et al*. 2010). Ubuntu can be installed as a dual boot with Windows or on a Windows 10 machine by downloading the Ubuntu application (Lloyd 2018).

STEP 1: pan-genome

This workflow is based on the use of Roary, a standalone pipeline allowing the calculation of a pan-genome. The installation is relatively easy but there are some requirements (Page *et al*. 2015; Sitto and Battistuzzi 2020). For beginners, we recommend to install Conda first and to work with Roary as a Bioconda package. To install conda, run the following command in the Linux Terminal (Grüning *et al*. 2018):

```
curl -O https://repo.anaconda.com/
miniconda/Miniconda3-latest-Linux-
x86_64.sh
sh Miniconda3-latest-Linux-x86_64.sh
```

Then one need to set up channels:

```
conda config --add channels r
conda config --add channels defaults
conda config --add channels bioconda
conda config --add channels conda-forge
```

And install Roary:

```
conda install roary
```

Roary requires the annotated assemblies in the GFF3 format and there are a few steps required to generate such files. We recommend that the GFF3 files

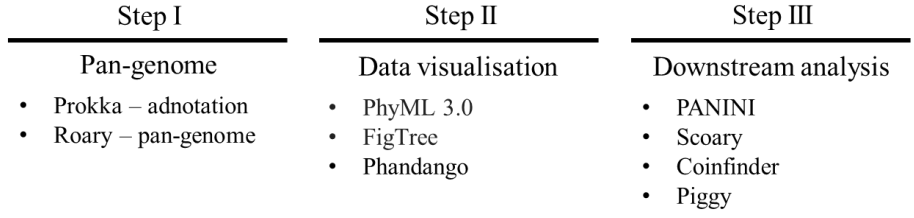

Figure 3. Scheme for a pan-genome analysis workflow.

be generated by Prokka (Seemann 2014, Page *et al*. 2015). The use of complete and finished genome sequences will give the best annotation results, but it is expected that the typical input will be a set of scaffold sequences. Prokka can be easily installed and used locally. Prokka is also available online within the Galaxy server (https://usegalaxy.org/); this approach might facilitate analysis in the case of beginners.

After installation of Roary, the user must optimise just a few parameters, but some additional options might be considered for more in-depth analysis. The simple command *roary *.gff* will run Roary with default parameters. The basic options to optimise the program are presented in Figure 4.

The major advantage of Roary is it is relatively simple to use and a pan-genome of even thousands of samples can be analysed on a standard desktop PC. In addition to the basic performance, Roary offers the *query_pan_genome* scripts, which perform set operations on the pan-genome to see the gene differences between groups of isolates (Sitto and Battistuzzi 2020).

Roary generates a set of output files, of which the most important and useful are (Sitto and Battistuzzi 2020):

- summary_statistics.txt – a text file that summarises the number of genes founded in the studied data, where genes are grouped into core, soft-core, shell and cloud based on the frequency within the studied genomes;
- gene_presence_absence.csv;
- gene_presence_absence.Rtab;
- accessory_binary_genes.fa.newick – a maximum likelihood tree generated based on the gene presence absence; and
- core_gene_alignment.aln – a file that contains the alignment of all core genes.

These files could be used for data visualisation as well as for downstream analysis with additional software.

```
Usage:   roary [options] *.gff

Options: -p INT    number of threads [1]
         -o STR    clusters output filename [clustered_proteins]
         -f STR    output directory [.]
         -e        create a multiFASTA alignment of core genes using PRANK
         -n        fast core gene alignment with MAFFT, use with -e
         -i        minimum percentage identity for blastp [95]
         -cd FLOAT percentage of isolates a gene must be in to be core [99]
         -qc       generate QC report with Kraken
         -k STR    path to Kraken database for QC, use with -qc
         -a        check dependancies and print versions
         -b STR    blastp executable [blastp]
         -c STR    mcl executable [mcl]
         -d STR    mcxdeblast executable [mcxdeblast]
         -g INT    maximum number of clusters [50000]
         -m STR    makeblastdb executable [makeblastdb]
         -r        create R plots, requires R and ggplot2
         -s        dont split paralogs
         -t INT    translation table [11]
         -ap       allow paralogs in core alignment
         -z        dont delete intermediate files
         -v        verbose output to STDOUT
         -w        print version and exit
         -y        add gene inference information to spreadsheet, doesnt work with -e
         -iv STR   Change the MCL inflation value [1.5]
         -h        this help message

Example: Quickly generate a core gene alignment using 8 threads
         roary -e --mafft -p 8 *.gff
```

Figure 4. List of Roary options available with the command *roary -h*.

STEP 2: data visualisation

Data obtained from Roary analysis could be easily visualised using local and online applications. The core_gene_ alignment.aln file could be used to generate the phylogenetic tree based on core gene single nucleotide polymorphisms (SNPs). This tree can be prepared by using the online version of PhyML 3.0 (http://www.atgc-montpellier.fr/phyml/) (Guindon *et al*. 2010), but it needs to be converted to the PHYLIP format. Trees based on accessory_binary_genes.fa. newick and core genes can be visualised and modified using FigTree (http://tree.bio.ed.ac.uk/software/figtree/) (Rambaut 2013). FigTree is designed as a graphical viewer of phylogenetic trees and as a program for producing publiccation-ready figures. It can be used on a desktop PC running Mac, Windows or Linux OS.

The gene_presence_absence.csv file together with any phylogenetic tree can be display using the Phandango website. One can simply drag and drop the Roary results into the web browser and then interactively play around with the data. Phandango can also be used to visualise the metadata for the samples, but it needs to be collected within a single file (csv format file). The sample IDs must be the same for all types of data used, or Phandango will not compile the data accurately (Hadfield *et al*. 2018).

The R script implemented in Roary allows one to generate two additional graphs: rarefaction curves, to conclude whether the analysed pan-genome is open or closed, and a plot of the number of new genes as a function of the number of studied sequences (Page *et al*. 2015; Sitto and Battistuzzi 2020).

STEP 3: downstream analysis

We propose the use of four additional software programs that will expand the pan-genome results obtained with Roary. These programs are the PANINI, Scoary, Coinfinder and Piggy pipelines.

PANINI

When the number of samples in a pan-genome analysis exceeds 100, one can use PANINI (Pangenome Neighbour Identification for Bacterial Populations). PANINI is a web-based tool that allows a user to identify the neighbours for each isolate in a data set. The tool is integrated with the Microreact platform for rapid online visualisation and exploration of pan-genomes, together with relevant epidemiological, geographical, temporal and other metadata (Abudahab *et al*. 2019).

The tool requires three types of input data: the gene_presence_absence.Rtab file (Roary output), any phylogenetic tree and a file containing the metadata of interest. After introducing the gene_presence_absence.Rtab file (drag and drop), the network file (DOT format) can be sent to Microreact when the other files are uploaded. The result will be displayed online, and Microreact allows a user to browse and to select data of interest (Abudahab *et al*. 2019).

The file containing metadata should be prepared in the csv format. It could include any relevant data. The geographical distribution of isolates could be displayed against a world map; this information must be specified by latitude and longitude columns. The file requires an ID column and each ID must be the same as in the other input files and must be unique. The metadata will be displayed against a phylogenetic tree comprised of coloured dots (default mode). The colour can be defined by user by the additional column maned: data_ name _colour. The colour must be defined by hex triplet number (Abudahab *et al*. 2019). The use of PANINI is intuitive and well described by the tool's authors, and there is also a video walkthrough.

Scoary

Roary output data could be used for a pan-genome wide association study. For this purpose, we recommend Scoary (Brynildsrud *et al*. 2016). Scoary is designed to take the gene_presence_absence.csv file from Roary and a traits file created by the user. It calculates the associations between all genes in the accessory genome and the traits. A traits file is a binary table (csv format) in which individual isolates are described with 1 or 0, sequentially with or without a trait. For example, when analysing the relationship between genetic diversity and the source of isolation, if the strain was isolated from a particular source of interest, the value is 1, while the remaining isolates receive the value 0 (Brynildsrud *et al*. 2016). The easiest way to install Scoary is with the pip package manager:

```
pip install scoary
```

The use of Scoary requires the following basic command:

```
scoary -g <gene_presence_absence.csv> -
t <traits.csv>
```

The user can also modify the parameters by additional flags, as shown at Figure 5.

Scoary outputs a single csv file per trait in the traits file. The file contains a list of genes with additional statistical characteristics. The output data need to be filtered manually. Candidate genes can be determined to be significantly related to a trait if they have achieved a 'naïve' p value < 0.05, a Benjamini-Hochberg corrected p value < 0.05 and an empirical p value < 0.05. A particular gene is considered to be positively related to a trait when the odds ratio is > 1 and to be negatively related when the odds ratio is < 1 (Espadinha *et al*. 2019, Touchon *et al*. 2020).

Coinfinder

Coinfinder allows one to assess the occurrence of interactions between genes in the pan-genome. The software tests for the occurrence of gene association and dissociation events among the accessory genes. The algorithm on which the program is based assumes the rejection of core genes and strongly unique genes to increase the precision of the analysis. The application uses the output (the gene_presence_absence.csv table) generated by Roars (Whelan *et al*. 2020). We recommend installing Coinfinder with Conda:

```
conda install -c defaults -c bioconda -
c conda-forge coinfinder
```

Coinfinder requires gene information (the gene_presence_absence.csv table) and a phylogeny as input. The phylogeny should be Newick formatted; we recommend using the core SNP-based phylogeny from the Roary output (Whelan *et al*. 2020). To run Coinfinder with default parameters, use the following line:

```
usage: scoary [-h] [-t TRAITS] [-g GENES] [-n NEWICKTREE] [-s START_COL]
              [--delimiter DELIMITER] [-r RESTRICT_TO] [-o OUTDIR] [-u]
              [-p P_VALUE_CUTOFF [P_VALUE_CUTOFF ...]]
              [-c [{I,B,BH,PW,EPW,P} [{I,B,BH,PW,EPW,P} ...]]] [-m MAX_HITS]
              [--include_input_columns GRABCOLS] [-w] [--no-time] [-e PERMUTE]
              [--no_pairwise] [--collapse] [--threads THREADS] [--test]
              [--citation] [--version]

Scoary version 1.6.16 - Screen pan-genome for trait-associated variants
```

Figure 5. List of Scoary options available with the command *scoary -h*.

```
./confinder [OPTIONS]
File input- specify either:
    -i or --input          The path to the gene_presence_absence.csv output from Roary
                           -or-
                           The path of the Alpha-to-Beta file with (alpha)(TAB)(beta)
    -I or --inputroary     Set if -i is in the gene_presence_absence.csv format from Roary
    -p or --phylogeny      Phylogeny of Betas in Newick format (required)
Max mode (mandatory for coincidence analysis):
    -a or --associate      Overlap; identify groups that tend to associate/co-occur (default).
    -d or --dissociate     Separation; identify groups that tend to dissociate/avoid.
Significance- specify:
    -L or --level          Specify the significnace level cutoff (default: 0.05)
Significance correction- specify:
    -m or --bonferroni     Bonferroni correction multiple correction (recommended & default)
    -n or --nocorrection   No correction, use value as-is
    -c or --fraction       (Connectivity analysis only) Use fraction rather than p-value
Alternative hypothesis- specify:
    -g or --greater        Greater (recommended & default)
    -l or --less           Less
    -t or --twotailed      Two-tailed
Miscellaneous:
    -x or --num_cores      The number of cores to use (default: 2)
    -v or --verbose        Verbose output.
    -r or --filter         Permit filtering of saturated and low-abundance data.
    -U or --upfilthreshold Upper filter threshold for high-abundance data filtering (default: 1.0 i.e. any alpha in >=100/% of betas.
    -F or --filthreshold   Threshold for low-abundance data filtering (default: 0.05 i.e. any alpha in <=5% of betas.
    -q or --query          Query a specific gene.
    -T or --test           Runs the test cases and exits.
    -E or --all            Outputs all results, regardless of significance.
Output:
    -o or --output         The prefix of all output files (default: coincident).
```

Figure 6. List of Coinfinder options available with the command *coinfinder -h*.

```
coinfinder -i <gene information> [-I] |-p <phylogeny> -o <output prefix> [--associate|--dissociate]
```

One might also change some options (see Figure 6).

Coinfinder produces a number of output files, with the default prefix of coincident_, which have been well described by (Whelan *et al*. 2020). The tool identifies pairs of associating/dissociating genes that are clustered in components or sets of genes that are related to each other. In addition, the results obtained with Coinfinder can be visualised using the Gephi graphics program. The produced charts should be interpreted as follows. Individual genes are represented by individual points on the plot (nodes). The lines connecting these points (edges) indicate the presence of interactions between the genes of the studied genomes. The groups of genes for which the presence of statistically significant correlations were found are depicted with different colours; these groups are called components. Genes within a given component show an association or dissociation. Occasional relationships between genes from different components can also be observed (Whelan *et al*. 2020).

Piggy

The above-mentioned tools allow for a detailed description of a pan-genome. All are focused on genes: their distribution, interaction and importance. To gain better insight into the phylogeny of a particular species, one can use the Piggy pipeline. Piggy works similarly to Roary, except it is focused on the intergenomic regions (termed IGRs) rather than genes. Piggy also detects and specifies highly divergent ('switched') intergenic regions (IGRs) upstream of genes. Similarly to Roary, IGRs can be described as core, present in all samples, accessory or unique. Based on a core IGR alignment file, a user can create the phylogenetic tree. Therefore, the use of this tool allows a user to understand not only the gene-based phylogeny, but also phylogeny based on non-coding regions. This information could be useful to better understand the evolution of tested strains. On the other hand, this tool provides insight into regions of genome sequences that might play a regulatory role (Thorpe *et al*. 2018).

Piggy can be easily obtained from github (https://github.com/), by a simple command line:

```
Piggy - version 1.5

--in_dir|-i      <STR>   input folder [default - current folder]
--out_dir|-o     <STR>   output folder [default - current folder/piggy_out]
--roary_dir|-r   <STR>   folder where roary output is stored [required]
--threads|-t     <INT>   threads [default - 1]
--nuc_id|-n      <INT>   min percentage nucleotide identity [default - 90]
--len_id|-l      <INT>   min percentage length identity [default - 90]
--edges|-e               keep IGRs at the edge of contigs [default - off]
--size|-s        <STR>   size of IGRs to extract [i-j] [default 30-1000]
--method|-m      <STR>   method for detecting switched IGRs [g - gene_pair, u - upstream] [default - g]
--R_plots|-R             make R plots (requires R, Rscript, ggplot2, reshape2) [default - off]
--fast|-f                fast mode (doesn't align IGRs or detect switched regions) [default - off]
--help|-h                help
--version|-v             version
```

Figure 7. List of Piggy options available with the command *piggy -h*.

```
git clone https://github.com/harry-thorpe/piggy.git
```

After cloning the Piggy repository, its directory should be either added to user $PATH or one can run Piggy by specifying its location in the terminal. For Piggy to work, Roary must be run first. The output folder produced by Roary is required as an input to Piggy. We recommend running Roary with the -s flag to keep paralogs together, so secondary Roary analysis can be performed (Thorpe *et al*. 2018).

To run Piggy, a user must specify the direction to files containing annotated assemblies in the GFF3 format, Roary output files and the output direction. The list of options are shown in Figure 7. Piggy produces several output files:

- cluster_intergenic_alignment_files,
- switched_region_alignment_files and
- IGR_presence_absence.csv.

The IGR_presence_absence.csv file can be visualised with Phandango, as can the gene_presence_absence.csv file generated by Roary. Piggy also generates two additional graphs, one presenting gene and IGR accumulation curves, and a histogram showing the frequency of genes and IGR regions identified within the tested set of samples (Thorpe *et al*. 2018).

Conclusions

The bacterial pan-genome is a relatively new concept for microbial genomics, but in recent years the number of studies focused on its investigation has increased rapidly. This approach allows one to better understand the diversity and evolution of bacteria, both at the inter- and intra-species levels. The results might help to improve taxonomy and ultimately lead to the development of more specific and sensitive methods of bacteria identification. On the other hand, knowing the relationship of the pan-genomic diversity of bacteria isolated from different sources (and/or time points) can be used as a basis to design new therapeutics. Hence, it is worth developing the area of pan-genome research.

Within this paper, we have reviewed some in silico tools that could be used by beginners who have an interest in pan-genome investigation. Each of them covers important features of pan-genome studies, namely core genes and pan-genome-based phylogeny, pan-genome-level diversity of strains, pan-genome wide association study and, in contrast to gene-focused studies, the diversity of non-coding sequences. When used in combination, the reviewed tools allow solid pan-genome investigation of bacterial species of interest.

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Comparison of the antioxidant potential of some herbal teas produced from ecological and traditional crops

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ABSTRACT

The growing public awareness of the dangers regarding chemicals used in traditional agriculture has led to consumers seeking valuable and contaminant-free products. Ecological agriculture has become synonymous with high health value and product safety. The aim of this study was to evaluate the antioxidant activity and the total polyphenolic content of infusions of herbal tea bags and loose teas from traditional crops, as well as infusions of loose teas from ecological crops. Raw material comprised dried flowers of *Matricaria chamomilla* and *Tilia cordata*, as well as dried leaves of *Urtica dioica*, *Melissa officinalis* and *Mentha piperita*. Herbal infusions were prepared using three brewing times: 5, 10 and 20 min. The analysis of antioxidant potential was performed using in vitro methods such as DPPH, ABTS and FRAP. The polyphenolic content was determined using the Folin-Ciocalteu method. The antioxidant activity of the studied tea infusion depended on the method by which the plants were cultivated and the brewing time. The ecological agriculture conditions seem not to stimulate the synthesis of antioxidants. However, the possibility to obtain other beneficial properties of the studied plants is an indication to carry out ecological cultivation.

KEYWORDS: free radicals, organic cultivation, antiradical activity, polyphenols content, oxidative stress

Introduction

Herbs have been widely used for a long time in folk and conventional medicine, human nutrition and cosmetics; therefore, great attention is paid to their beneficial properties resulting from the content of active compounds (Parkash *et* al. 2018). A myriad of biologically active substances can be found in herbal raw materials, including essential oils, organic acids, vitamins, phenolic compounds and their derivatives, tannins and mucilages (Kohlmünzer et al. 2007;

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Kazimierczak *et al*. 2011; Parkash *et al*. 2018). Plants with regenerative, anti-inflammatory, calming, diuretic, disinfecting, mental-performance-enhancing and fatigue-preventing activities are frequently applied in traditional medicine.

Plant infusions for home use are prepared by pouring hot water over flowers, leaves or the whole herb. Commercially available herbal teas consist of a mixture of ground and dried raw materials derived from traditional or ecological crops (Pełczyński *et al*. 1993, Gulumian *et al*. 2018, Jiang *et al*. 2018, Kędzia *et al*. 2018). The growing public awareness of the dangers regarding chemicals used in traditional agriculture has resulted in consumers seeking whole-some and contaminant-free products.

The aim of ecological crops, aside from obtaining high-quality products, is to keep the environment clean and to maintain appropriate soil parameters without contamination. Therefore, ecological agriculture has become synonymous with high health value and product safety (Rasul *et al*. 2004; Magdoff 2007; Reganold *et al*. 2016; Kazimierczak *et al*. 2017; Zargoosh *et al*. 2019; Piotrowski *et al*. 2020). This is explained by the theory of carbon-nitrogen balance, suggesting that a higher content of substances with antioxidant properties can be found in plants from ecological crops. Plants fertilised with nitrogen compounds would produce more compounds such as peptides, amino acids, proteins and alkalids, in contrast to plants grown with ecological fertilisers, which would produce more nitrogen-free compounds, including carbohydrates, phenolic compounds and their derivatives, vitamin C or other antioxidants (Reganold *et al*. 2016; Kazimierczak *et al*. 2017, Zargoosh *et al*. 2019).

The aim of this study was to evaluate the antioxidant potential and the total polyphenolic content of infusions of herbal tea bags and loose teas from traditional crops, as well as infusions of loose teas from ecological crops.

Materials and methods

Raw material and infusion preparation

Herbal teas used in this study consisted of dried flowers of *Matricaria chamomilla*, and *Tilia cordata*, as well as dried leaves of *Urtica dioica*, *Melissa officinalis* and *Mentha piperita*. For this purpose, herbal teas commonly purchased by consumers were selected. Infusions of three different variants of the teas were examined in terms of the type of production – loose teas from ecological crops versus loose teas and tea bags from traditional crops. The following herbal material have been evaluated: Dary natury – batch number 01012022; Zakład Zielarski Kawon-Hurt – batch number 012020; and Herbapol – batch number 02102018.

Herbal infusions with the same content of plant material were prepared using boiling tap water and three brewing times – the average brewing time recommended by the producers (10 min), half as long (5 min) and twice as long (20 min).

Chemicals and methods

2,2-Diphenyl-1-picrylhydrazyl (DPPH), 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (Trolox), 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ) and 2,2'-azobis (3-ethylbenzothiazoline-6-sulphoniac acid) (ABTS) were purchased from Sigma Aldrich (St. Louis, MO, USA). The Folin-Ciocalteu (F-C) reagent, gallic acid (GA), and iron(II) sulfate heptahydrate were supplied by Merck (Darmstadt, Germany); anhydrous sodium carbonate by Loba Chemie (Mumbai, India); and

iron(III) chloride hexahydrate, hydrochloric acid 36%, sodium acetate anhydrous, potassium persulfate, acetic acid 99.5% by Chempur (Piekary Śląskie, Poland). All reagents were of analytical grade.

The antioxidant potential of the infusions was assessed by in vitro methods including DPPH, ferric ion reducing antioxidant power assay (FRAP) and ABTS techniques as described previously (Muzykiewicz *et al*. 2017, 2018). The polyphenolic content was determined using the F-C method (Muzykiewicz *et al*. 2017).

In the case of the FRAP method, the results are presented as mg $FeSO_4$/g raw material. Trolox was used as a reference substance in the DPPH and ABTS methods and gallic acid was in used the F-C method.

Antioxidant activity is expressed as the per cent radical scavenging activity (% RSA) for the DPPH and ABTS methods, mg $FeSO_4$/g of raw material for the FRAP method and mg GA/g of plant material for the F-C method.

The differences between the antioxidant activity (assessed by the DPPH, ABTS, FRAP and F-C methods) were statistically analysed by Wilcoxon's signed-rank test (parameter z). Pearson's correlation coefficients (r) were determined between the antioxidant activity of infusions assessed using individual methods. The results were calculated using Statistica 12PL software and are presented as the arithmetic means ± standard deviations (SD).

Results

All studied infusions showed antioxidant activity; for most of the applies methods, the *M. officinalis* infusions had the highest antioxidant activity. The *T. cordata* infusions also showed high activity using the DPPH method and the *M. piperita* infusions showed high activity with the ABTS method. The *M. chamomilla* infusions had the lowest antioxidant potential regardless of the assessment method.

Figures 1 and 2 present the free radical scavenging activity (% RSA) of the studied infusions. In the case of the DPPH method, the results ranged from 26.55% ± 0.33% (*U. dioica* leaf infusion) to 77.68% ± 1.34% (*T. cordata* flower infusion). For the ABTS method, the values ranged from 27.37% ± 1.50% (*M. chamomilla* flower infusion) to 99.64% ± 0.25% (*M. piperita* leaf infusion).

M. officinalis leaf infusions had the highest reducing potential assessed by the FRAP method (14.42 ± 0.36 mg $FeSO_4$/g of raw material) while *M. chamomilla* had the lowest (1.22 ± 0.17 mg $FeSO_4$/g of raw material) (Figure 3).

There were similar trends for the total polyphenolic content: this parameter ranged from 0.56 ± 0.08 to 4.64 ± 0.06 mg GA/g of raw material (Figure 4).

The brewing time had an inconsistent effect on the antioxidant activity of the infusions. *M. chamomilla*, *M. officinalis*, *M. piperita* and *T. cordata* brewed for the longest time (20 min) showed higher antioxidant activity while *U. dioica* brewed for the shortest time (5 min) had the highest antioxidant activity.

In the majority of the cases, infusions from traditional crops had greater antioxidant properties: tea bags in the case of the ABTS (28.03% ± 1.70% to 98.79% ± 0.16% RSA) and the FRAP (1.22 ± 0.17 to 14.42 ± 0.36 mg $FeSO_4$/g of raw material) methods, and loose teas for the DPPH (26.55% ± 0.33% to 77.68% ± 1.34% RSA) and the F-C (1.44 ± 0.02 to 4.64 ± 0.06 mg GA/g of raw material) methods (Figures 1–4). For most of the studied herbs, the infusions of herbs declared as ecological had the lowest values. Moreover, almost all of the infusions from these plants also showed the lowest polyphenolic content

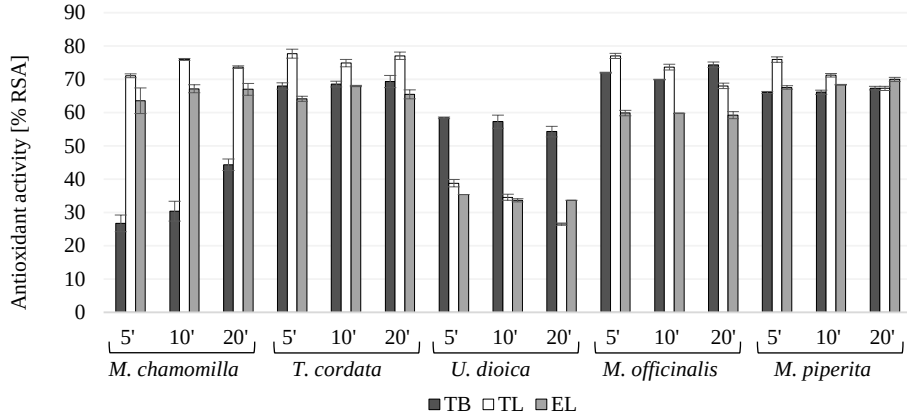

Figure 1. The mean antioxidant potential ([% RSA] – radical scavenging activity) of herbal tea infusions of different materials – tea bags (TB) and loose tea (TL) from traditional crops and loose tea from ecological crops (EL) – evaluated with the DPPH method. The error bars represent the standard deviation (SD).

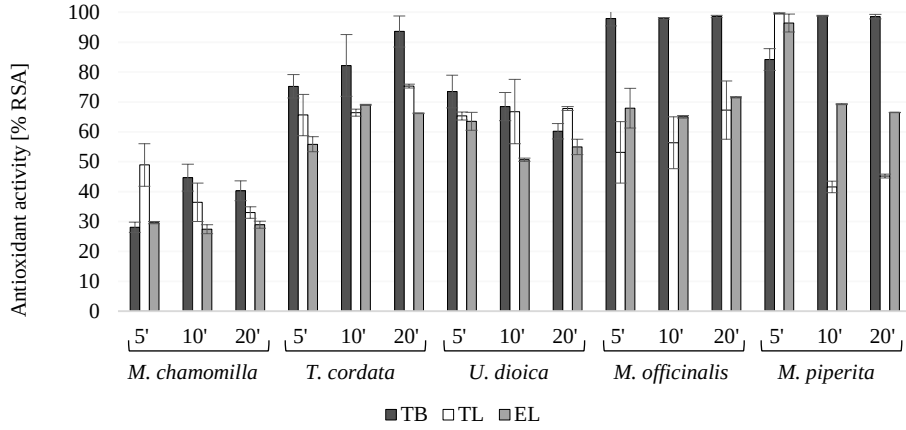

Figure 2. The mean antioxidant potential ([% RSA] – radical scavenging activity) of herbal tea infusions of different materials – tea bags (TB) and loose tea (TL) from traditional crops and loose tea from ecological crops (EL) – evaluated with the ABTS method. The error bars represent the standard deviation (SD).

as assessed by the F-C method, between 0.56 ± 0.02 and 2.63 ± 0.13 mg GA/g of raw material (Figure 4).

The Pearson correlation coefficients (r) between the antioxidant activities measured with each method are provided in Table 1. The highest correlations for all tea infusions were between the ABTS and F-C methods ($r = 0.575$, $p < 0.001$) and between the ABTS and FRAP methods ($r = 0.573$, $p < 0.001$).

Based on Wilcoxon's signed-rank test, the difference in the activity of infusions made of tea from traditional crops (tea bags and loose tea) and ecological loose tea was statistically significant ($p < 0.010$): tea bags versus ecological loose tea ($z = 3.865$) and loose tea versus ecological loose tea ($z = 3.291$).

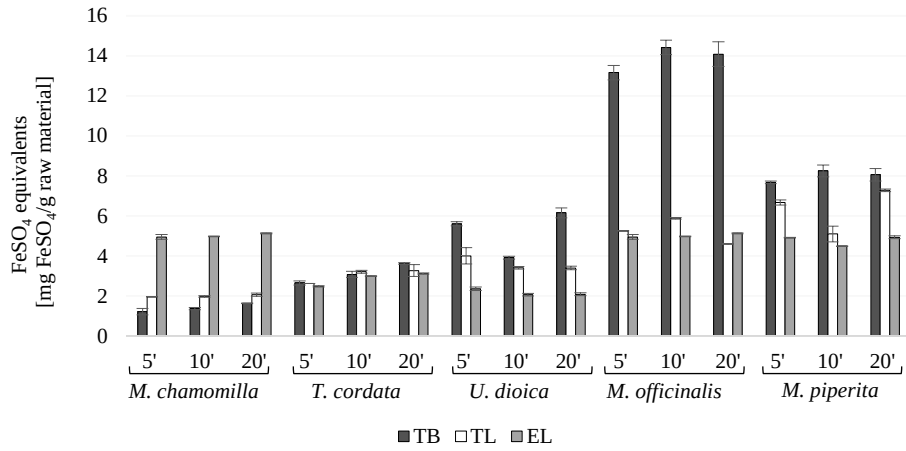

Figure 3. The mean antioxidant potential of herbal tea infusions of different materials – tea bags (TB) and loose tea (TL) from traditional crops and loose tea from ecological crops (EL) – evaluated with the FRAP method (ferric ion reducing antioxidant power assay), expressed as $FeSO_4$ equivalents [mg $FeSO_4$/g raw material]. The error bars represent the standard deviation (SD)

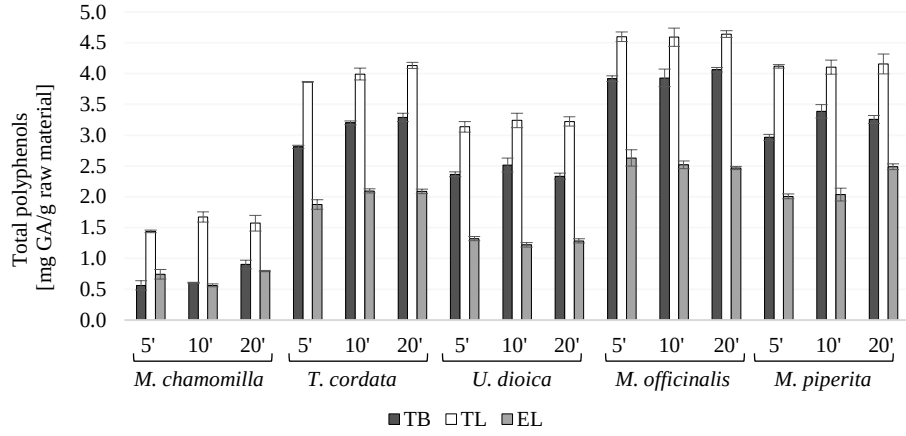

Figure 4. The mean polyphenolic content in herbal tea infusions of different materials – tea bags (TB) and loose tea (TL) from traditional crops and loose tea (EL) from ecological crops – evaluated with the Folin-Ciocalteu method and expressed as gallic acid equivalents [mg GA/g raw material]. The error bars represent the standard deviation (SD)

Discussion

Some researchers suggest that plants exposed to environmental stress activate antioxidant defense systems, which helps to maintain the structural integrity of their cellular components and possibly reduces oxidative damage (Caverzan *et al*. 2016). Many studies have confirmed the presence of bioactive compounds, particularly antioxidants, in plants from organic crops (Kazimierczak *et al*. 2011).

It has been proven that removing pesticides and synthetic fertilisers in ecological agriculture promotes the production of secondary metabolites, such as phenolic compounds and their

derivatives. In the presence of nitrogen-containing compounds, plants produce more alkaloids and amino acids. On the other hand, if the concentration of nitrogen is lower, as in ecological plants due to the use of organic fertilisers, carbon compounds, including mono- and polysaccharides, some vitamins and polyphenols, could be produced primarily (Hallmann *et al*. 2007; Kazimierczak *et al*. 2011; Reganold *et al*. 2016).

Kazimierczak *et al*. (2011) compared the content of bioactive substances in different spice plants from organic and conventional crops. In their study, plants from traditional crops contained more vitamin C and total flavonoids, but a lower concentration of phenolic acids compared with the organic crops. Rembiałkowska *et al*. (2003) assessed the nutritional value of two varieties of tomatoes from conventional and ecological cultivation. They showed that tomatoes from ecological crops contained less vitamin C and lycopene but more beta-carotene and flavonoids belonging to the group of polyphenols (Rembiałkowska *et al*. 2003). The same authors came to similar conclusions after evaluating red onions from ecological and conventional cultivation. In the case of onions from ecological crops, there were more flavonoids, vitamin C and anthocyanins (Hallmann *et al*. 2007). Kapoulas *et al*. (2019) observed no difference in the total polyphenolic content in lettuce and green onions depending on the production system. In their study, the examined vegetables were fertilised with agricultural chemicals or organic fertilisers.

In our study, the antiradical capacities of herbal tea infusions from traditional and ecological crops were compared. The results obtained with the DPPH, ABTS, FRAP, and F-C methods indicated the highest potential for infusions of traditionally cultivated herbs. In addition, infusions from traditionally cultivated teas had higher total polyphenolic content compared with the infusions from ecological teas. The plants used to produce ecological teas were harvested from their natural state in pollution-free certified zones or were grown on ecological farms. The lower antioxidant activity of the studied infusions from ecological teas may be due partly to the weather conditions during the cultivation (Grabowska *et al*. 2016). The herbs used by different tea producers were likely harvested at different times. Śmiechowska *et al*. (2011) suggest that the economic crisis has resulted in the use of fewer agrochemicals, which, in their study, resulted in almost the same heavy metal contamination of plants from both types of crops.

In our study, the *M. officinalis* infusions showed the highest free radical scavenging properties. Kazimierczak *et al*. (2017) characterised the same herb as having a high content and variety of flavonoids.

Summary

Infusions of the studied, frequently used herbal teas showed high antioxidant properties. The antioxidant potential and total polyphenolic content depended on the method of cultivation, raw material used and the brewing time. The infusions of the selected teas, particularly M. officinalis, may be a valuable source of antioxidants to be used to prevent the harmful effects of free radicals. In the case of the studied herbs, ecological agriculture does not seem to stimulate the synthesis of antioxidants. However, the possibility to obtain other beneficial properties of the studied plants could be an indication to carry out this type of cultivation.

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Cultivation of oyster mushrooms (*Pleurotus* sp.) using organic waste: an example with *Pleurotus pulmonarius* (Fr.) Quel.

BIOOPEN 2021 – POST-CONFERENCE ARTICLE

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ABSTRACT

Pleurotus pulmonarius (Fr.) Quel. is a mushroom species that occurs widely in nature on all continents except Antarctica. It is most common in North America. Its fruiting bodies are characterised by a mild taste and a slight anise aroma. These mushrooms are valued as a source of nutrients and substances with a healing effect. The anticancer, anti-inflammatory and antioxidant properties of *P. pulmonarius* have been scientifically proven, as well as its strong antihyperglycemic activity. *P. pulmonarius* is easy to grow because it has a very aggressive mycelium towards cellulose-containing materials. In Poland, it can be grown on substrates based on cereal straw and various types of organic waste, including agricultural, horticultural, textile and forestry. In intensive crops, the substrates are also enriched with protein and carbohydrates. On an industrial scale, *P. pulmonarius* is grown primarily in Asia and North America on locally available organic materials.

KEYWORDS: edible and medicinal mushrooms, mushroom cultivation, substrate, supplementation, fruiting body

Introduction

Pleurotus pulmonarius (Fr.) Quel. (Figure 1) belongs to the kingdom *Fungi*, the phylum *Basidiomycota*, the class *Agaricomycetes*, the order *Agaricales* and the family *Pleurotaceae*. In the literature, it has been called 'boczniak płucny', its former Polish name, as well as 'lung oyster mushroom' and 'phoenix oyster mushroom' (Croan 2004, Jonathan *et al*. 2012). *P. pulmonarius* has a small hat, 5–20 cm in size, most often cream or light brown in colour. It is shell shaped and paddle shaped – in younger specimens it is oyster-shaped – with a bent, sometimes ruffled edge. The gills have the colour of a hat, they are very dense and narrow and converge deeply to the stem, which is unremarkable. This species of oyster mushroom has a distinct stem, which grows on trees, reaching up to 6 cm in height and up to 1.5 cm in thickness. It is lateral, thinner towards the

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Figure 1. Fruiting bodies of *Pleurotus pulmonarius.*

base, whitish and felt. At first, the flesh is firm and juicy, hard and dry with age, with an imperceptible smell and sweet taste. The smell emitted by *P. pulmonarius* is indistinct and resembles anise, and its taste is slightly sweet and mild (Baggio *et al*. 2010; Janitor *et al*. 2007; Lechner *et al*. 2004; Škubla 2008).

In natural conditions, *P. pulmonarius* is found on all continents except Antarctica. It is most commonly found in North America. It is a species that grows in single specimens or in dense clumps of deciduous trees. It has also been observed on conifers. In the natural environment under Central European conditions, *P. pulmonarius* is found on dead trunks, logs and carps of trees such as beeches, oaks, birches, lindens, poplars and willows. It often occurs in the form of groups consisting of a dozen larger and smaller specimens that grow out of a common base or are arranged in a tile-like pattern on top of each other (Vilgalys *et al*. 1996; Janitor *et al*. 2007; Trudell and Ammirati 2009).

P. pulmonarius produces fruiting bodies during the warm season, from summer (June) to early autumn (October) (Škubla 2008). In Poland, it is a rare species and is included in the Red List of Plants and Mushrooms of Poland. It has the status of V – the species is at risk of extinction (Wojewoda and Ławrynowicz 2006).

P. pulmonarius may be confused with *Pleurotus ostreatus* (Jacq.) P. Kumm.; however, it has larger and thicker gray-blue fruiting bodies, non-yellowing gills and appears only in late autumn. It can also be confused with *Pleurotus dryinus* (Pers.) P. Kumm., which produces larger fruiting bodies and grows mostly singly (Siwulski and Sobieralski 2004; Škubla 2008).

The aim of this work was to characterise *P. pulmonarius* and the methods of cultivating mushrooms from the genus *Pleurotus*.

Nutritional value and healing properties of *P. pulmonarius*

P. pulmonarius is a tasty edible mushroom. In addition to its culinary value, its fruiting bodies have high nutritional value – they contain, among

others, highly digestible proteins, fibre, vitamins and minerals, as well as biologically active substances with proven pro-health properties. The biological activity of these mushrooms has been confirmed in numerous laboratory and clinical tests, which have demonstrated, among others, their antibacterial, antiviral, antifungal, antitumor, immunomodulatory, antiallergic, anti-inflammatory, anti-atherosclerotic, hepatoprotective, blood sugar and cholesterol lowering effects. *P. pulmonarius* is low in calories due to the low content of lipids and starch. In many countries it is considered a healthy food (Manzi and Pizzoferrato 2000; Jose *et al*. 2002; Wasser 2002; Badole *et al*. 2006; Bernaś *et al*. 2006; Badole and Bodhankar 2007; Thekkut-tuparambil and Kainoor 2007; Smiderle *et al*. 2008; Akkanni *et al*. 2010; Lavi *et al*. 2010a, 2010b; Ramesh and Pattar 2010; Adebayo *et al*. 2012; Baggio *et al*. 2012; Olufemi *et al*. 2012; Patel *et al*. 2012; Smiderle *et al*. 2012; Xu *et al*. 2012).

Cultivation of *P. pulmonarius*

In recent years there has been a very dynamic development regarding the production of edible and medicinal mushrooms around the world. This development has mainly been due to the high availability of inexpensive, often waste materials from agricultural production and the wood and textile industry, which can be a potential substrate for their cultivation. Poland, compared with the other European Union countries, is still an ecologically clean area. Therefore, it has enormous possibilities regarding the use of substrates not contaminated with heavy metals and pesticides for mushroom cultivation (Siwulski and Sobieralski 2004).

P. pulmonarius is the second most widely cultivated species in the world after P. ostreatus (Chiu et al. 1998). These two oyster mushrooms are grown in a similar way and are most sold to consumers. *P. pulmonarius* was first found by an scientist in India and then brought to China, where its cultivation began. Recently, there has been an increase in the importance of its cultivation in Japan (Yatsuzuka *et al*. 2007). *P. pulmonarius* is especially popular in Western Europe, North America and New Zealand (Baggio *et al*. 2010). In Nigeria, it is one of the most consumed species (Onuoha *et al*. 2009).

P. pulmonarius is a saprotroph, a heterotrophic organism that obtains energy from the decomposition of organic compounds from the remains of dead higher organisms. Oyster mushrooms, thanks to their ability to decompose cellulose and lignin, cause white rot of wood. Therefore, they belong to the group of parasites living on trees (Gerhardt 2006, Trudell and Ammirati 2009).

P. pulmonarius is an easy-to-grow mushroom because it has a very aggressive mycelium towards materials containing lignin-cellulose. Researchers have shown that it can be grown on a variety of locally available waste such as: chopped cocoa pods, cotton waste, chopped corn straw, palm oil waste, tobacco straw, tea leaves, rice straw, sugar cane pomace, waste paper and sawdust (Banjo *et al*. 2004, Adebayo *et al*. 2009, Akinfemi and Ogunwale 2012). Adebayo *et al*. (2009) conducted research on the use of cotton waste substrate with the addition of cassava peel for the cultivation of *P. pulmonarius*. They showed that it can be used for mushroom cultivation if supplemented with a good source of nitrogen, such as urea or soybeans. Onuoha *et al*. (2009) used different substrates for the cultivation of *P. pulmonarius*. They found that sawdust was the best growth medium for this species, and the addition

addition of cassava peel had a positive effect on the growth of the mushrooms.

Jonathan *et al*. (2012) showed that *P. pulmonarius* significantly supports the breakdown of lignin in agricultural waste (sorghum and rice straw stalks), a finding that is of great importance due to the possibility of using them as animal feed. Agricultural wastes are mostly fibrous and have low nutritional value due to the content of lignin, cellulose and hemicellulose, and the low levels of protein, soluble carbohydrates and minerals (Jonathan *et al*. 2010; Olusola and Anslem 2010). *P. pulmonarius* is able to bioconvert a wide variety of lignocellulosic materials by secreting extracellular enzymes (Jonathan *et al*. 2012). Silva *et al*. (2002) proved a similar effect of *P. pulmonarius* on waste from cotton cultivation, which can then be used as animal feed. Moreover, Valdez *et al*. (2008) confirmed the possibility of using agricultural waste from the cultivation of *P. pulmonarius* as feed for ruminants. This species, grown on wheat straw, changes its chemical composition and improves its nutritional value. Akinfemi and Ogunwale (2012) obtained similar results when cultivating *P. pulmonarius* on rice straw. The mushroom improved the digestibility of straw for ruminants and its nutritional value.

In Poland, oyster mushrooms are cultivated on 'hard straw' – wheat, triticale and rye, which, after preliminary preparation and inoculation with mycelium, are packed into bags made of perforated foil. In intensive, large-scale cultivation, the substrates are also enriched with protein and carbohydrates. This process utilises agricultural products such as chaff, bran, cereal grain, meal and oilseed cake, as well as other materials rich in nitrogenous substances and sugars (Figures 2 and 3) (Siwulski and Sobieralski 2004).

P. pulmonarius tolerates summer temperatures during mycelium growth and yielding. Fruiting bodies are formed at 10–20 °C and develop at 12–24 °C. Light is an important factor that determines the yield and morphological features of oyster mushroom fruiting bodies. The best effects are obtained by using lighting with an intensity of 500

Figure 2. Fruiting bodies of *Pleurotus pulmonarius* in large-scale cultivation.

Figure 3. Large-scale cultivation of oyster mushrooms (from the genus *Pleurotus*) on a farm.

and 700 lx for 14 h a day (Siwulski and Sobieralski 2004; Siwulski *et al*. 2012).

Amateurs can also cultivate *P. pulmonarius*. This process is carried out under covers on the wood of practically all deciduous species and on sawdust from pine, spruce and fir trees, chopped straw and straw bales. Enrichment of substrates in amateur cultivation is not recommended. Substrates that are too rich in readily available nutrients are often attacked by moulds and bacteria that can inhibit or completely prevent the growth of the *P. pulmonarius* mycelium. The cultivation can also be carried out in open areas, in shady places (Siwulski and Sobieralski 2004; Siwulski *et al*. 2012).

Summary

P. pulmonarius is a species that requires careful evaluation. Currently, it is practically unknown in Poland and not cultivated on a large scale. *P. pulmonarius* can be an alternative to producers of other oyster mushroom species and at the same time diversify the market offerings of edible mushrooms because it can withstand high temperatures during mycelium growth and yielding. On the other hand, the management of waste in its production will significantly increase the profitability of cultivation and reduce its costs. Its cultivation will also provide a new way of using problematic waste. The development of a cheap and simple method for *P. pulmonarius* production in Poland may contribute to the introduction of this species to large-scale cultivation. Understanding the dependence of morphological and qualitative characteristics on the type of substrate and growing conditions will allow for the optimisation of production to obtain high-quality fruiting bodies, which will be suitable for human consumption and which could be used as raw material to obtaining biologically active substances.

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Aetiology, prophylaxis and management of preeclampsia

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ABSTRACT

Although preeclampsia affects approximately 3%–8% of pregnancies worldwide and is a major contributor to maternal and neonatal mortality and morbidity, the aetiology of preeclampsia is still not fully understood. This review presents the current knowledge on the aetiology of preeclampsia, with a special emphasis on risk factors and their role, and describes recommendations for the prevention and treatment of preeclampsia.

KEYWORDS: proteinuria, gestational hypertension, trophoblast invasion, uteroplacental malperfusion, endothelial dysfunction

Introduction

Preeclampsia (PE) is defined as de novo gestational hypertension after the 20th week of pregnancy (>140/90 mmHg) with new-onset proteinuria (>300 mg/24 h) or at least one other sign of maternal organ dysfunction (haematological complications, kidney and liver dysfunction, neurological complications such as eclampsia) or uteroplacental dysfunction (such as foetal growth restriction) (Braunthal and Brateanu, 2019; Tomimatsu *et al*. 2019). The course progressively worsens, potentially leading to maternal and foetal death. Although the clinical signs of PE resolve after delivery, PE causes persistent disruption of maternal and foetal physiology. PE has long-term effects on both the mother and child, causing increased susceptibility to hypertension and chronic kidney disease (Turbeville and Sasser; 2020). Women after a pregnancy complicated by PE are more likely to have hypertension, renal dysfunction and cardiovascular and cerebrovascular diseases (Benschop *et al*. 2019).

Although PE affects approximately 3%–8% of pregnancies worldwide (Aouache *et al*. 2018) and is a major contributor to maternal and neonatal mortality and morbidity (Geldenhuys *et al*. 2018), the aetiology of this disorder is still not fully understood. A two-stage model of PE pathogenesis is currently accepted (Figure 1). The first stage, uteroplacental malperfusion, leads to the second stage, in which cycles of ischaemia-reperfusion in the placenta trigger the release of cytokines, anti-angiogenic factors and reactive oxygen

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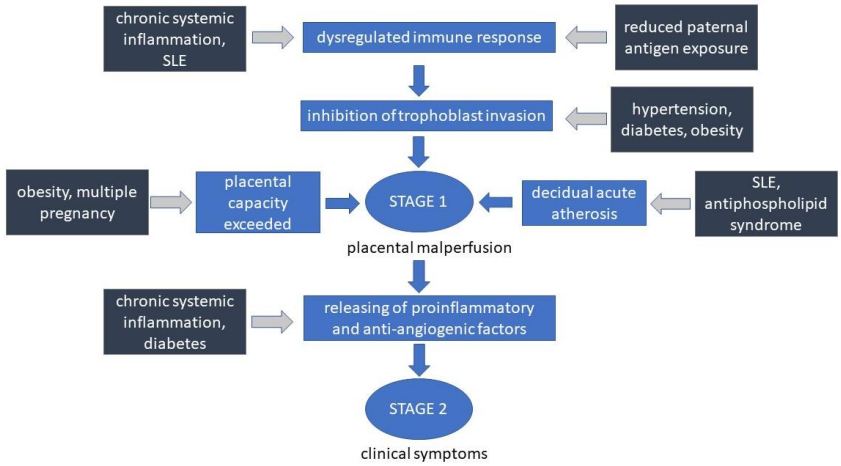

Figure 1. The two-stage model of preeclampsia pathogenesis. The risk factors may affect the trophoblast invasion of spiral arteries, conducive to decidual acute atherosis and exceeded placental capacity, which lead to Stage 1. The reduced uteroplacental perfusion induces placental release of proinflammatory and anti-angiogenic factors into the maternal circulation, leading to systemic vascular dysfunction, Stage 2. Pre-existing maternal risk factors such as diabetes and chronic systemic inflammation also predispose to vascular dysfunction. SLE, systemic lupus erythematosus.

species (ROS). When these molecules reach the maternal circulation, they can cause maternal endothelial dysfunction and systemic inflammation. Systemic maternal disease is associated with clinical symptoms of PE and is proposed as a second stage of PE pathogenesis model (Staff 2019).

Stage 1: inhibition of trophoblast invasion

The process of extracellular trophoblast invasion (EVT) that occurs after embryo implantation is essential for normal placental development. After implantation of the embryo into the maternal endometrium, the blastocyst grows into an inner cell mass and an outer trophectoderm. The inner cell mass forms the embryo, while the outer cell mass gives rise to the primary, secondary and tertiary villi. These villi form the structural basis of the placenta. Trophoblasts differentiate into two main cell lineages: villous and extravillous. The EVT participate in the process of attachment of the placenta to the uterine wall and in the remodelling of the maternal spiral artery (Li *et al*., 2021).

There are many risk factors that may affect the trophoblast invasion of the spiral arteries and may lead to the vascular dysfunction observed in PE (Figure 2). Epidemiologic observations regarding the risk of PE include the first pregnancy with a given partner, conception early in a new relationship, contraception using barrier methods and donor egg pregnancies. Moreover, oral exposure to the father's semen can be protective against PE in a subsequent pregnancy (Kenny and Kell, 2017). These risk factors suggest the involvement of reduced paternal antigen exposure in the pathogenesis of PE.

Maternal interactions with paternal alloantigens induce local maternal-foetal immunotolerance. Regulatory T cells (Tregs) have been acknowledged as the most important cells involved in the prevention of immune-mediated rejection of the semiallogenic foetus (Gobert and

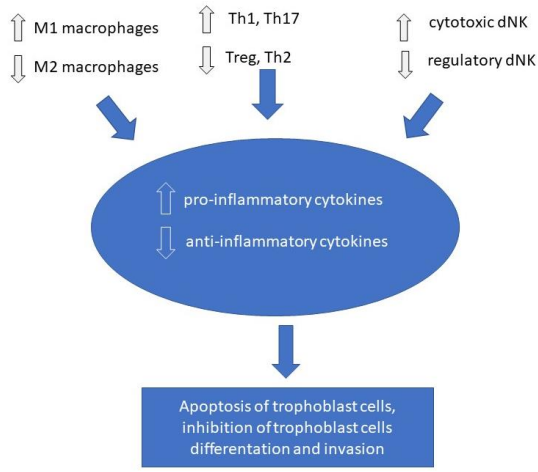

Figure 2. The dysregulation of the immune system in preeclampsia is associated with an imbalance of pro-inflammatory and anti-inflammatory cytokines. The plethora of pro-inflammatory immune cells promote inflammation. The reduced count of anti-inflammatory cells is not efficient in suppression the activation and proliferation of proinflammatory cells subsets. dNK, decidual natural killer cells; Th, helper T cell; Treg, regulatory T cell.

Lafaille 2012). Transforming growth factor-β (TGF-β) and prostaglandin E, constituents of seminal fluid, act as Treg-inducing agents (Kenny and Kell 2017). The fact that over 20% of pregnant women with systemic lupus erythematosus (SLE) have pregnancies complicated by PE confirms the important role of Tregs in pregnancy. SLE is an autoimmune disease that is associated with immune alterations, especially with a reduction in Tregs (Amaral *et al*. 2017). Due to anti-inflammatory properties, Tregs ensure protection against inflamematory injury via suppression of the activation and proliferation of proinflammatory cell subsets (Lu and Hu 2019). The protective role of Tregs has been shown in a reduced uterine perfusion pressure (RUPP) rat model (Cornelius 2018). This model closely mimics the hypertension, systemic and renal vasoconstriction, oxidative stress and immune system dysregulation in the mother, and intrauterine growth restriction in the offspring (Li *et al*. 2012). Transfer of Tregs from normal pregnant rats into RUPP rats reduced both blood pressure and levels of inflammatory cytokines and attenuated the occurrence of foetal growth restriction. The Treg transfer was associated with increased levels of anti-inflammatory cytokines, reduction of oxidative stress and downregulation of placental vasoconstrictor endothelin 1 (ET-1) (Cornelius 2018). A reduction in the number of Tregs or their function may lead to incorrect trophoblast invasion and unfavourable spiral artery remodelling (Gobert and Lafaille 2012; Staff 2019). Depletion of decidual Tregs resulted in increased trophoblast apoptosis and disturbed the invasion of extravillous trophoblasts into the decidua (Cornelius 2018). Previous pregnancy with the same father generates memory Tregs, a phenomenon that reduces the risk of PE in subsequent pregnancy. However, the level of Tregs seems to decline over time, and thus the protective effect of previous pregnancies disappears when there is long time period between pregnancies (Staff 2019).

There is dysregulation of the immune system in preeclamptic pregnancies. Figure 2 provides a summary of the main components of a dysregulated immune system in PE. Immunological imbalance occurs due to elevation of following subsets of proinflammatory T helper cells: Th17 and Th1 and reduction of Th2 and Treg subsets (Geldenhuys *et al*. 2018). Proinflammatory helper T cell subsets are linked to chronic systemic and local placental inflammation in PE. Activated Th1 and Th17 cells secrete proinflammatory cytokines including tumour necrosis factor α (TNF-α) and interleukins (ILs), namely IL-6 and IL-17. TNF-α signalling results in endothelial cell activation, reduction of nitric oxide synthase (NOS) and elevation of vasoconstrictor ET-1. IL-6 has also been shown to mediate the expression of endothelin and it plays a role in endothelial permeability. IL-17 plays a role in the pathophysiology of PE via generation of placental oxidative stress, which stimulates neutrophils, leading to the release of ROS. In addition, IL-17 stimulates B cells to produce agonistic autoantibodies to the angiotensin II type I receptor (AT1-AA) (Cornelius 2018). Activation of the AT1 receptor by AT1-AA results in the inhibition of trophoblast invasiveness (Aouache *et al*. 2018). Stimulation of AT1 is also associated with the release of antiangiogenic factors ET-1 and soluble vascular endothelial growth factor receptor 1 (sFlt1) (Lu and Hu, 2019). In RUPP rats, AT1-AA inhibition reduced blood pressure, improved renal function, and diminished circulating factors associated with PE, such as ET-1 and sFlt1 (Cunningham *et al*. 2019). Excessive secretion of sFlt1 is also associated with several PE risk factors, such as diabetes mellitus and gestational hypertension (Geldenhuys *et al*. 2018; Tomimatsu *et al*., 2019). sFlt1 affects normal endothelial function by reducing the available placental growth factor (PIGF) and vascular endothelial growth factor (VEGF) levels; these growth factors are essential for angiogenesis. Excess sFlt-1 leads to inhibition of extravillous trophoblast invasion and differentiation and subsequently contributes to the poor placentation (Staff 2019).

Uterine natural killer (uNK) cells have regulatory functions in addition to the classical killing role of NK cells (recognition and destruction of infected cells and cancer cells). There are two types of uNK cells observed in the uterus. In the nonpregnant uterus, the uNK cells are known as endometrial NK (eNK) cells whereas those in the pregnant uterus are termed decidual NK (dNK) cells. The latter are phenotypically and functionally different from NK cells in the peripheral circulation. They have unique functions such as the production of cytokines, chemokines and growth factors, and they participate in all steps of placentation including trophoblast invasion into the maternal endometrium and vascular remodelling (Jabrane-Ferrat and Siewiera 2014). This is associated with the features of the decidual microenvironment characterised by physiological hypoxia in the placenta and regulation by hormones (progesterone, oestrogen) and trophoblast-derived soluble factors (like soluble HLA-G). The phenotype of dNK cells can be modified by severe inflammation in the uterine microenvironment caused by severe stress, autoimmune diseases and infections. In PE, dNK cells shift from a regulatory to a cytotoxic phenotype. This alteration is associated with elevated production of interferon γ (IFN-γ), causing apoptosis of trophoblasts that invade the developing spiral arteries (Geldenhuys *et al*. 2018). Maternal uNK cells with a regulatory pheno-

type are associated with reduced PE risk in the next gestation. uNK cells may develop trained memory after a first pregnancy, potentially promoting more efficient placentation in subsequent pregnancies. These cells are essential for proper uterospiral artery evolvement. Interactions between killer-cell immune-globulin-like receptor, expressed on the uNK cells, and foetal HLA-C proteins, expressed on invading trophoblasts, are important for the placentation process. It has also been proposed that uNK cells interact with Tregs to facilitate vascular remodelling. In addition, it has been demonstrated using myometrial sections that spiral arteries of parous uteri sustain some of the remodelling patterns that occur during pregnancy, a phenomenon that could explain the reduced risk of PE in parous women and the general increase in birth weights in subsequent pregnancies (Staff 2019).

In the decidua, two types of macrophages are prevalent: M1 macrophages with proinflammatory properties, phagocytic and microbicidal functions, and M2 macrophages with immunosuppressive properties that support maintenance of immunological homeostasis during pregnancy. M2 macrophages regulate inflam-mation by producing immune-suppressive cytokines (such as IL-10 and TGF-β1), inducing the expression of Tregs and inhibiting the cytotoxic function of dNK cells and phagocytosing apoptotic trophoblasts. Furthermore, M2 cells promote placentation via secretion of factors associated with tissue remodelling and angiogenesis (like VEGF, PIGF). The decreased level of immunosuppressive cytokines observed in PE is associated with a reduced M2 macrophage count (Geldenhuys *et al*. 2018). In PE, lower levels of IL-10 have been observed; this anti-inflammatory cytokine has important functions during pregnancy. Thanks to its ability to inhibit the secretion of inflammatory cytokines, it provides an important counterbalance for inflammation at the foetal-maternal interface and is responsible for stimulating the differentiation of Tregs from naïve T cells (Cornelius 2018). Augmented inflammation then induces proinflammatory M1 macro-phages, which increase apoptosis of cytotrophoblasts (Geldenhuys *et al*. 2018).

Stage 2: causes of uteroplacental malperfusion

Uteroplacental malperfusion can occur without poor placentation, when the placenta outgrows the uterine capacity (placental compression). Women who are obese and have had multiple pregnancies are more likely to have larger placentas, and they also have an increased risk of developing PE. It can cause the terminal villi to be compressed, a factor that impedes intervillous perfusion and causes syncytiotrophoblast hypoxia and oxidative stress. This process triggers a maternal response that is similar to defective uteroplacental artery remodelling and the release of harmful molecules derived from the placenta. Maternal factors (e.g. chronic arterial disease, obesity and some autoimmune diseases) may impact multiple aspects of placentation, placental size and placental function, in addition to amplifying maternal vascular sensitivity to factors shed by the placenta to generate the maternal clinical signs. Maternal autoimmune diseases are associated with excessive decidual inflammation. This process could predispose a woman to develop decidual acute atherosis at any stage of pregnancy (Staff 2019). Acute atherosis is a vascular change of the placenta characterised by lipid-filled foam cell accumulation, lymphocytic infiltration and fibrinoid

and fibrinoid necrosis (similarly to atherosclerosis) (Kim and Kim 2015). It is also associated with spiral artery thrombosis and can worsen placental intervillous perfusion. Decidual acute atherosis has been observed in women with concurrent systemic lupus erythematosus and antiphospholipid syndrome, suggesting that massive pre-pregnancy vascular inflammation observed in autoimmune diseases may be associated with the generation of inflammatory arterial pregnancy lesions (Staff 2019). Antiphospholipid antibodies (aPLs) are able to induce the formation of thrombi in the vasculature and activate cells involved in haemostasis, including platelets, endothelial cells and monocytes. aPLs also inhibit fibrinolysis, which leads to placental thrombosis (Lu and Hu 2019). Pregestational obesity may disrupt the arterial architecture in the placenta and affect the contraction and relaxation capacity of the placenta. Placentas in obese woman generate excessive mitochondrial ROS (due to defective respiratory chain), exacerbating the imbalance between free radicals and antioxidants (Alcala *et al*. 2018).

Stage 3: maternal systemic inflammation and vascular endothelial dysfunction

Persistent episodes of hypoxia-reoxygenation caused by defective placentation can injure the villi and generate oxidative stress (OS) in placental tissue (Staff 2019). The syncytiotrophoblast (the outer layer of the trophoblast) is notably sensitive to ROS because of insufficient concentrations of antioxidative enzymes like manganese superoxide dismutase (Cornelius 2018). Placental malperfusion has also been linked to increased placental endoplasmic reticulum (ER) stress and activation of the unfolded protein response (Staff 2019). During PE, the syncytiotrophoblast experiences accelerated ageing with upregulation of the apoptotic cascade, necrotic breakdown with release of necrotic debris and an increase in syncytial aggregates. The syncytiotrophoblast cells secrete sensecence-associated beta-galactosidase (SAβ-Gal) and express proapoptotic p53 and cyclin dependent kinases (CDKs) inhibitors at a higher rate, indicating cessation of cell cycle and senescence. It has been proposed that senescence in placental cells can be a result of excessive ROS accumulation and ER stress. The pro-inflammatory senescence-associated secretory phenotype (SASP) is observed with production of SASP proteins, which then activate the cyclooxygenase pathway and enhance the generation of proinflammatory factors (cytokines and chemokines) (Manna *et al*. 2019). Syncytiotrophoblast stress, associated with excessive trophoblast senescence or other causes of trophoblast dysfunction, leads to the release of increased levels of inflammatory factors into the maternal circulation. Even a relatively low degree of syncytiotrophoblast stress could be sufficient to reach stage 2 of PE development, especially in women who are susceptible to excessive inflammatory substances, such as women with prior chronic vascular inflammation, or with pregnancy-related excessive vascular inflammation, such as in gestational diabetes mellitus. The more excessively inflamed the maternal vasculature is, the less inflammatory stimuli from the placenta are needed to reach the clinical stage (Staff 2019).

Defective placentation causes the release of multiple particles form trophoblasts. These placental-derived factors act directly on the maternal vascular endothelium or act indirectly by increasing OS and stimulating the release of pro-inflammatory cytokines and vasoactive compounds (Alcala *et al*. 2018). The placenta as a source of circulating

vasoactive factors causes widespread systemic maternal vascular dysfunction. It is associated with the stimulation of prostaglandin synthesis, activation of the renin-angiotensin system, the release of various anti-angiogenic factors, altered synthesis of gasotransmitters and vasoconstrictors and changes in reactivity to vasoactive factors (Tong and Giussani 2019). The vascular endothelium comprises a single layer of epithelial cells overlaying the interior surface of blood vessels. This layer acts as a semi-selective barrier between the vessel lumen and the more exterior layers of the vessel wall. The cells have paracrine and autocrine functions, allowing them to modulate arterial vasomotility, leucocyte adhesion and extravasation, proliferation and differentiation of smooth muscle building more exterior layer of the vessel wall, and platelet coagulation and fibrinolysis (Aouache *et al*. 2018). The endothelium plays a key role in modulating vascular tone by releasing a variety of endo-thelium-derived relaxing factors (such as vasodilator prostaglandins, NO) and contracting factors. The direct contact of epithelial cells with the blood flow makes them sensitive to circulating factors. Endothelial dysfunction is characterised by reduced production or action of these relaxing mediators (Godo and Shimo-kawa 2017). Secretion of trophoblast-derived angiogenic factors – for example, sFlt1 and soluble endoglin (sEng) – cause anti-angiogenic imbalance that is characteristic of PE. sFlt1 represents an important link between placental dysfunction (Stage 1) and the maternal symptoms (Stage 2). sFlt1 affects endothelial function by reducing available PIGF and VEGF in placental and maternal cells (by binding to them and blocking their ability to act on the endothelium) (Staff 2019). VEGF enables vasodilation by stimulating the NO-cyclic guanosine monophosphate vascular relaxation pathway and by increasing calcium ion (Ca^{2+}) production. Ca^{2+} binds to endothelial nitric oxide synthase (eNOS) and increases eNOS activity, enhancing NO production from L-arginine (Geldenhuys *et al*. 2018). Therefore, due to a reduction in available VEGF, the NO concentration also decreases. NO may also be reduced due to decreased expression of NO-synthesising enzymes or due to NO reactions with free radicals (Tong and Giussani 2019). The systemic inflammatory response in PE results in high amounts of ROS, produced by activated blood cells. The accumulation of ROS produced by blood cells and released from hypoxic placental cells is a reason for systemic oxidative stress with high production of ROS and reactive nitrogen species (RNS). ROS may react with NO, causing a lower bio-availability of NO (Mannaerts *et al*. 2018). The superoxide anion reacts with NO to form peroxynitrite, a powerful oxidising agent that can initiate lipid peroxidation, among other functions (Taravati and Tohidi 2018). In addition, peroxynitrite promotes the production of the vasoconstrictor ET-1 (Aouache *et al*. 2018). Increased sEng contributes to endothelial dysfunction by the inhibition of TGF-β1 signalling. This pathway is involved in angiogenesis by regulating VEGF expression. The inhibition of TGF-β1 signalling will reduce endothelium-dependent vasodilation and increase endothelial cell apoptosis (Geldenhuys *et al*. 2018). TNF-α and other circulating factors present in the plasma of preeclamptic woman can induce oxidative stress in epithelial cells indirectly by upregulating the receptors associated with oxidised low-density lipoprotein (LDL) uptake. AT1-AA can activate the AT1 receptor in arterial epithelial and smooth muscle cells as

well as in renal mesangial cells. In vivo experiments in pregnant rats showed that administration of AT1-AA induces PE symptoms, including hypertension and proteinuria (Aouache *et al*. 2018). The mechanisms leading to endothelial dysfunction and reduced vasodilation are summarised in Figure 3. Disturbances in endothelial homeostasis lead to a pro-inflammatory, vasoconstrictive and prothrombotic tendency of endothelial cells (Mannaerts *et al*. 2018). Clinically, these changes are associated with vascular resistance and increase in arterial blood pressure in preeclamptic woman and proteinuria due to acceleration of glomerular endotheliosis (Manna *et al*. 2019).

Prophylaxis and management

The National Institute for Health and Clinical Excellence (NICE) has proposed a classification of the risk factors for PE. This grading distinguishes moderate and high risk factors to allow defining the group of patients for whom the immediate application of prophylactic measures should be applied. The high-risk factors are: hypertensive disorders in previous pregnancies, chronic arterial hypertension, chronic kidney disease, autoimmune diseases and diabetes (type 1 or 2). Moderate risk factors include: primiparity, inter-delivery interval > 10 years, multiple gestations, body mass index (BMI) > 35 kg/m2, advanced age (> 40 years old) and a family history of PE. The presence of two moderate risk factors or a single high risk factor indicates that prophylactic measures should be implemented. The American College of Obstetricians and Gynecologists (ACOG) has reported the same risk factors but has classified them all as high risk. The only exception is the BMI factor >30 kg/m^2 (Mayrink *et al*. 2018). To prevent the occurrence of PE, the International Society for the Study o f Hypertension in Pregnancy (ISSHP) recommends that women at high risk of

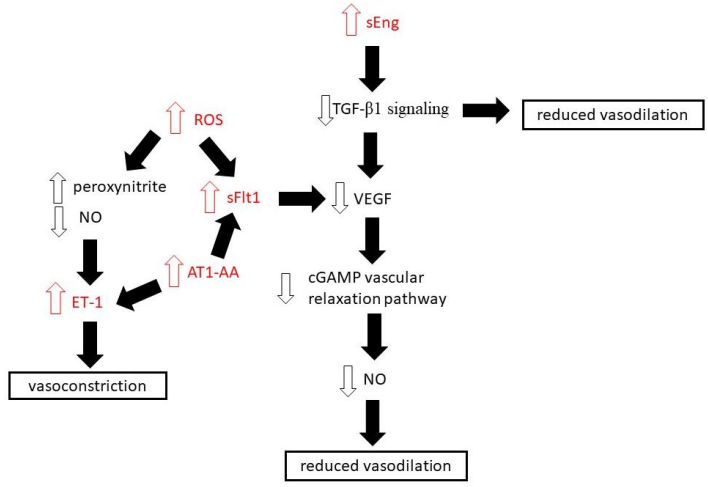

Figure 3. Mechanisms leading to endothelial dysfunction and reduced vasodilation in preeclampsia. Placental-derived factors – sEng, sFlt1, AT1-AA and ROS – contribute to a massive systemic endothelial dysfunction and vasoconstriction. AT1-AA, agonistic autoantibodies to the angiotensin II type 1 receptor; cGAMP, cyclic guanosine monophosphate–adenosine monophosphate; ET-1, endothelin 1; ROS, reactive oxygen species; sENG, soluble endoglin; sFlt1, soluble vascular endothelial growth factor receptor-1; TGF-β1, transforming growth factor beta-1; VEGF, vascular endothelial growth factor.

PE should be treated (preferably before 16 weeks but definitely before 20 weeks) with low-dose aspirin. In addition, women considered at increased risk should receive supplemental calcium (1.2–2.5 g/day) if their calcium intake is likely to be low (< 600 mg/day) (Brown *et al*. 2018; Braunthal and Brateanu 2019).

Currently, there is no effective treatment for PE and the only effective management is termination of pregnancy. The aim of clinical management of PE is to prevent maternal and foetal mortality. The most important elements of management are: tight control of maternal and foetal conditions, treatment of hypertensive emergency and prevention of maternal seizures (Amaral *et al*. 2017). Maternal monitoring should include blood pressure monitoring, evaluation for proteinuria (if it is not already present), blood tests for haemoglobin, platelet count, liver transaminases, creatinine and uric acid (Brown *et al*. 2018). If treatment fails to compensate for severe maternal hypertension or if severe complications of pregnancy are present, preterm delivery is recommended. To prevent adverse consequences for the foetus, it is necessary to optimise the time of delivery (Amaral *et al*. 2017). The ISSHP advises that women with PE should be induced to deliver their baby if they have reached 37 weeks of gestation or if they have developed complications such as: severe hypertension resistant to treatment, pulmonary oedema, progressive thrombocytopaenia, worsening renal and liver disorders, visual disturbances, convulsions and non-reassuring foetal status (suspected foetal hypoxia) (Brown *et al*. 2018). Corticosteroids (betame-thasone, dexamethasone) are recom-mended if preterm labour is suspected in a woman with PE or if preterm termination of pregnancy is considered. Corticosteroid treatment should be given for 7 days before preterm delivery (Fox *et al*. 2019) to accelerate structural maturation of the lungs (by stimulating surfactant phospholipid production in alveolar cells). Recognition and manage-ment of persistently elevated (> 15 min duration) severe hypertension is neces-sary to prevent a hypertensive crisis, which is associated with life-threatening complications such as eclampsia. This is accomplished by aggressive treatment of systolic blood pressure (SBP) ≥ 160 mmHg and/or diastolic blood pressure (DBP) ≥ 105 mmHg. Intravenous labetalol or hydralazine treatment is considered the first-line therapy for an acute hypertensive emergency. Oral nifedipine may also be used as a first-line therapy, especially when intravenous access is not available. Emphasis is put on avoiding excessive lowering of blood pressure because it may further decrease placental perfusion and potentiate negative effects on the foetal condition. The goal of treatment is to lower the maternal blood pressure to a SBP of 140–150 mmHg and a DBP of 90–100 mmHg. Prophylaxis against maternal seizures (eclampsia) is achieved by the use of magnesium sulphate. This anticonvulsant is administered intravenously or intramuscularly (Amaral *et al*. 2017). Magnesium sulphate is also used if preterm termination of pregnancy is planned before 32 weeks of gestation due to its proven neuroprotective effects in neonates (Fox *et al*. 2019). Platelet transfusion is recommended in patients with platelets < 50,000/μl before caesarean section or when the platelet count is ≤ 20,000–25,000/μl before vaginal delivery to prevent excessive bleeding during delivery (Lam and Dierking 2017). In the early postpartum period, women with PE should be considered at high risk for preeclamptic complications (eclamptic seizures may develop for the first time in the early postpartum period). The patient's condition should be

monitored at least every 4 h for at least 3 days after delivery (Brown *et al*. 2018).

Conclusions

Although PE is one of the leading causes of maternal and neonatal morbidity and mortality worldwide, due to limited understanding of its aetiology, there is still no effective therapy for this condition. At present, the only effective treatment is termination of pregnancy. The aim of clinical management of PE is to prevent maternal and foetal mortality. The most important elements of management are the treatment of emergency conditions and the prevention of maternal seizures. Although the clinical symptoms of PE resolve after delivery, PE causes permanent disruption to maternal and foetal physiology. Further research into the pathogenesis of PE is needed to find an effective therapy.

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