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CONTENTS

MINIREVIEW
Use of plant extracts to control and treat AB, enterotoxin-related diarrhea
KOMIAZYK M., PALCZEWSKA M., SITKIEWICZ I., GROVES P. 3

ORIGINAL PAPERS
Quantitative expression of Candida albicans aspartyl proteinase genes SAP7, SAP8, SAP9, SAP10 in human serum in vitro
STANISZEWSKA M., BONDARYK M., MALEWSKI T., KURZĄTKOWSKI W. 15

The effect of oleanolic and ursolic acids on the hemolytic properties and biofilm formation of Listeria monocytogenes
KUREK A., MARKOWSKA K., GRUDNIAK A.M., JANISZOWSKA W., WOLSKA K.I. 21

Iron shortage and bile salts play a major role in the expression of ompK gene in Vibrio anguillarum
HAMOD M.A., BHOWMICK P.P., SHUKUR Y.N., KARUNASAGAR I., KARUNASAGAR I. 27

Development of symbiotics with inulin, palatinose, α-cyclodextrin and probiotic bacteria
PRANCKUTĖ R., KASIAJNIENĖ T., MIŠKIENĖ N., CITAVIČIUS D. 33

Uropathogenic Escherichia coli isolates with different virulence genes content exhibit similar pathologic influence on vero cells
OBAID J.M.A.S., MANSOUR S.R., EL SHAHEDY M.S., RABIE T.E., AZAB A.M.H. 43

Novobiocin sensitivity of Salmonella typhimurium dam and/or seqA mutants
CHATTI A., ALOUI M., TAGOUIRTI J., MIHOUB M., LANDOUlsi A. 51

The antimicrobial susceptibility of Helicobacter pylori strains isolated from children and adults with primary infection in the lower silesia region, Poland
GOSCINIAK G., BIERNAT M., GRABINSKA J., BIŃKOWSKA A., PONIEJEWIERKA E., IWAŃCZAK B. 57

Helicobacter pylori infection in type 1 diabetes children and adolescents using 13C urea breath test
CHOBOT A., BĄK-DRABIK K., SKAŁA-ZAMOROWSKA E., KRZYWICKA A., KWIATKOWSKA J., POŁAŃSKA J. 63

The utility of Caco-2 cells in isolation of enteroviruses from environmental and clinical material
WIECZOREK M., CIAJKA A., WITEK A., LITWINSKA B. 69

Psychrotrophic lipase producers from arctic soil and sediment samples

Plant growth promotion rhizobacteria in onion production
ČOLO J., HAJNAL-JAFARI T.I., ĐURIĆ S., STAMENOV D., HAMIDOVIĆ S. 83

The prevalence of Anaplasma phagocytophilum in questing Ixodes ricinus ticks in SW Poland
KIEWRA D., ZALESKI G., CZUŁOWSKA A. 89

Biocontrol efficacy of different isolates of Trichoderma against soil borne pathogen Rhizoctonia solani

Prevalence of EBV genotypes in Polish, Taiwanese and Arabic healthy students and association between genotypes and 30-bp deletion in the LMP-1 gene phylogenetic analysis
POLZ D., PODSIADŁO Ł., STEC A., POLZ-DACEWICZ M. 105

SHORT COMMUNICATIONS
Microbial transformations of 3-methoxyflavone by strains of Aspergillus niger
KOSTRZEWA-SUSŁOW E., DYMARSKA M., JANECZKO T. 111

Zeaxanthin biosynthesis by members of the genus Muricauda
SUDHARSHAN PRABHU, REKHA P.D., ARUN A.B. 115

INSTRUCTION FOR AUTHORS
Submission of manuscripts: http://pjm.indexcopernicus.com/
Instructions for authors: http://www.pjm.microbiology.pl/
1. Introduction

The World Health Organization (WHO) prepares yearly reports on the global status of diarrheal diseases (http://www.who.int/topics/diarrhoea/en/). In addition to reporting statistics for the number of cases and fatalities, the WHO identifies actions to reduce the spread of disease and improve treatments. In developed, rich countries, diarrhea is usually associated with poor hygiene standards in the food industry but in poor countries the majority of cases are related to a lack of clean water supplies and underdeveloped medical networks. Diarrheal symptoms can be caused by multiple inflammatory bowel diseases such as ulcerative colitis, Crohn's disease or irritable bowel syndrome. However, symptoms can also be caused by a wide range of bacteria (Campylobacter sp., Clostridium sp., Staphylococcus aureus, Vibrio cholerae, Shigella dysenteriae, enterotoxigenic strains of Escherichia coli), viruses (rotaviruses, noroviruses) and parasites (Giardia lamblia, Entamoeba histolytica and Cryptosporidium sp.). Because of the difficulties in diagnosis and the need of rapid therapy to prevent water and ion loss, rehydration therapy is used as a first line of action.

Oral Rehydration Therapy (ORT) provides a simple and rapid treatment that can be given by any adult to a suffering individual, either adult or a child, to improve recovery from diarrhea. ORT solutions are usually composed of water, sodium chloride, glucose, potassium ions and citrate and the formulation currently recommended by the WHO has an osmolarity of 245 mmol/L (Anonymous, 2002). In contrast, intravenous rehydration requires a nurse while drugs require a doctor and a significant period of time to start working. It must be stressed that ORT alleviates the symptoms and aids recovery but does not cure the source of the diarrheal episode. The treatment of diarrhea depends on the initial source, which often is not known. It has been suggested that the use of antibiotics for the treatment of diarrhea worsens the problem in cases related to infections with bacteria carrying phages encoding endotoxins (Prins, 1994). Therefore, ORT is of particular importance where the infectious agent has already been removed by the diarrheal episode.

Edible plants are traditionally used by many societies to alleviate and cure diarrhea. The properties of traditional plant extracts are worth exploring as they can stop or kill bacterial growth, neutralize or deactivate enterotoxins, are cheap and readily available, can give a better flavor to ORT, provide useful microelements and vitamins, and last, but not least, edible plants do not require the same level of extensive testing or
regulatory approval as new drugs. This review covers the scientific literature relevant to the use of plant extracts to treat enterotoxic bacterial disease, particularly diarrhea caused by the AB$_5$ family of enterotoxins (V. cholerae, S. dysenteriae and enterotoxin producing strains of E. coli). Together, the organisms that produce AB$_5$ enterotoxins result in more than 200 million episodes of diarrhea per year and around 2 million deaths. The plants reviewed here provide potential leads for improving ORT formulations and properties.

2. Mode of action of enterotoxic bacteria

AB$_5$ enterotoxins are originally encoded by prophages STX1 or STX2 of Shigella sp. (Herold, 2005), or by phage CTXφ of Vibrio sp. (McCloud, 2004). Strains of the bacteria that do not express enterotoxins, which are a majority, are usually benign. Phages can undergo horizontal transfer and infect/transfer the toxin to other bacterial species and this is the case for the STX1 and STX2 prophages with E. coli. The primary role of AB$_5$ molecules is not known. Recently it’s been suggested that AB$_5$ enterotoxin is a weapon against protozoa and immune system cells such as nucleophiles and their production is activated by reactive oxygen species.

Enterotoxic bacterial infection proceeds from the ingestion of contaminated water or food. Sufficient numbers of bacteria must survive passage through the stomach and into the intestine, where they anchor to the intestinal wall. Here, they colonize the intestinal wall and grow without any great ill-effects to the host. Once the bacterial colony reaches a certain number of cells, enterotoxin production is switched on. The assembled enterotoxin is secreted via the type II secretion system. Recent developments in research on strategies of V. cholerae to maintain fitness in different ecological niches and protein production are described in a recent review by Sikora (Sikora, 2013).

Cholera is a model bacterial disease caused by microorganisms producing AB$_5$-type enterotoxins (Beddoe et al., 2010). The molecular structure of the toxin is formed from a proper toxin – A subunit and donut-like ring of five B-subunits, Figure 1. The B-subunits, which recognize GM$_1$ gangliosides in human cells, act as a chaperone/delivery vehicle for the A-subunit.

Fig. 1. Primary and 3D structural data for cholera toxin (PDB accession code 1XTC).

A) Protein sequence of cholera toxin subunit A. The first 18 residues (italics) constitute a signal sequence that is removed during secretion. The A1 domain is separated from the A2 domain by ‘nicking’ – an exogenous human protease breaks the backbone chain between residue Ser212 and Met213. The A2 domain is denoted in italics and bold text. The A1 and A2 remain joined through a Cys-Cys linkage denoted by a line. B) Protein sequence of the cholera toxin subunit B. The signal peptide (italics) is removed during secretion from V. cholerae. C) Structure of cholera toxin (PDB: 1XTC) as a cartoon representation of the secondary structure elements of cholera toxin. The five B-subunits are colored in green-blue shades. The A2 subunit (magenta) forms a long helical element that is embed in the center of the donut-shaped B-subunit pentamer. The A2 subunit is linked to the A1 subunit (yellow) through disulfide bonds. B) schematic representation of the enterotoxin structure. The GM$_1$-binding sites are situated in the B-subunits and on the opposite face of the A1 subunit.
that sits on the opposite face of the donut. The B-sub-
units of different enterotoxic bacteria have similar sec-
condary and tertiary structures, as well as similar modes
of binding to GM₁, although their primary amino acid
sequences are quite distinct. The A-subunit is formed
from two polypeptide chains: a long alpha-helical A2
domain that anchors the A1 domain into the hole of
the B5 structure. The A1 domain, crosslinked to the
A2 domain through disulfide bonds, is processed in the
endoplasmic reticulum and released to the cytoplasm.

The introduction of the toxin to human cells fol-
sows a specific path involving initial endocytosis of the
GM₁-toxin complex followed by transport to the Golgi
and endoplasmic reticulum. The toxin is processed
in the endoplasmic reticulum and the A1 subunit is
released into the cytoplasm where subunit A1 binds
to, and activates, ADP-ribosylation factor 6 (Arf6).
This unwanted stimulation interferes with a range of
cellular processes involving cAMP. In particular, the
disregulation of cAMP levels results in the opening of
ion channels and rapid loss of ions and water from the
human cell which forms the diarrheal episode (Kopic
and Geibel, 2010).

The multi-step process of the bacterial life cycle,
involvement of a bacteriophage, toxin production,
transport and action provides several potential tar-
gets where the disease process can be mediated/modi-
fied. Plant extracts can inhibit the initial anchoring
of bacteria to the digestive system (Birdi et al., 2010),
down-regulate toxin production in bacteria (Birdi
et al., 2010; Brijesh et al., 2009), inhibit the binding of
toxin to GM₁ (Birdi et al., 2010; J.-C. Chen et al., 2006;
2007; 2009), close toxin-activated ion channels (Fischer
et al., 2004) etc.

Despite this, the majority of the reported research
focuses almost exclusively on the antimicrobial prop-
erties of plants (Table I). However, it needs to be noted
that concentration of studied antimicrobial plant
extracts is usually much higher and expressed as mini-
mal inhibitory concentration (MIC) in mg/ml range
than those observed for antibiotics – usually in µg/ml
(http://www.eucast.org/mic_distributions/).

Plants have been used as a treatment and source of
active substances for millennia with Ayurvedic prac-
tice and the roots of Chinese medicine providing the
best established, documented and known examples.
Ethnopharmacists collect the remaining local folk
knowledge in various worldwide regions such as India
(Dey and De, 2012; Tetali et al., 2009), Indonesia (Gros-
venor, Supriono and Gray, 1995) or Nigeria (Tekwu,
Pieme and Beng, 2012). In the majority of cases, little
or no scientific validation has been provided for the
efficacy of plant extracts against disease. Experienced
practitioners of plant medicine take careful note on the
method of collection and storage of the plant material,
as well as the preparation and use of the plant extract.
Still, there is often a lack of validated identification
of the bacterial species, treatment process, plant used,
or use of placebo controls. This is best exemplified by
making a simple internet search that often results in
general list of diseases that can be cured by a particular
plant, very often without any useful details on how to
prepare or use the plant material.

Scientists have validated the beneficial effects of
many plant extracts, including those against enterotoxic
bacteria, particularly the more common and readily
available plants. The antimicrobial active ingredients
are often hydrophobic in nature and are most efficiently
obtained by organic solvent extraction such as ethanol,
methanol or acetone requiring a chemical laboratory
(e.g. Rajan, Thirunalsundari and Jeeva, 2011). Data are
also collected for simple aqueous extracts or decoctions
– basically equivalent to a cup of herbal tea or broth.
While ethanol extracts might support the effects of
alcoholic beverages popularly used as digestives, they
are clearly unsuitable for children less than five years
old who represent one of the most sensitive groups
of diarrhea patients. The use of methanol or acetone
as a solvent raises safety issues. While the production
of essential oils through steam distillation is an energy
and time consuming process, unwanted solvents can
be avoided and a safer product can be produced. They
also potentially work in much lower concentrations
then aqueous extracts and so they make an interesting
alternative. Still, in our opinion, the ideal, active plant
extracts should be available from a simple cold or hot
water extract. Considering this point, the reviewed lit-
erature is focused towards the use of aqueous extracts.

3. Examples of active plants, plant parts
and mode of preparation

Folk medicine provides the starting point for many
investigations and the selection of plants and part of
the plants to study. However, we must recognize that
the properties of plants that have been cultivated and
selected over the millennia may change. For example
1000 year old Anglo-Saxon recipes were used to test
the antibacterial properties of plants such as Potentilla
reptans (European cinquefoil) against wound infections
and modern experiments found that plant extracts have
stronger antimicrobial properties against gram negative
intestinal bacteria such as E. coli than wound-infecting
bacteria (S. aureus) (Watkins et al., 2012). This makes
the extracts useful for a possible treatment of diarrhea,
and maybe less suitable in their original use to treat
skin lesions.

Indian Ayurvedic medicine is based on phytome-
dical properties practiced over several millennia.
Studies on Ayurvedic recipes revealed that an aqueous
Table I
Summary of antimicrobial properties of various plant extracts

<table>
<thead>
<tr>
<th>Region</th>
<th>Plant name</th>
<th>Method of preparation</th>
<th>Tested enteropathogens</th>
<th>Assay</th>
<th>Result</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Africa</td>
<td><em>Albizia gummifera</em></td>
<td>Hexane, ethyl acetate and methanol (MeOH) extracts</td>
<td><em>E. coli</em> LMP0101U, <em>S. dysenteriae</em> LMP0208U, <em>S. flexneri</em> LMP0313U</td>
<td>Agar disc diffusion</td>
<td>The MIC is dependent on the solvents and is between 0.032 (MeOH N. latifoli bark extr.) and 0.512 mg/ml.</td>
<td>Tekwu et al., 2012</td>
</tr>
<tr>
<td></td>
<td><em>Ficus exasperata</em></td>
<td>methanol, acetone, ethanol and boiling water extracts</td>
<td><em>V. cholerae</em>, <em>E. coli</em> ATCC 35218, <em>Shigella dysenteriae</em>, <em>Shigella flexneri</em>, <em>Shigella sonnei</em>, <em>Shigella boydii</em></td>
<td>Agar-well diffusion, Broth microdilution (MIC)</td>
<td>The MIC is dependent on plant extract preparation and bacterial strain. All of the plants have antimicrobial activities 0.039–0.312 mg/ml against <em>V. cholerae</em>, <em>S. dysenteriae</em> and <em>S. flexneri</em>. <em>E. coli</em> is resistant to <em>E. burkii</em>, <em>E. elephantina</em>, <em>Gymnosporia senegalensis</em>, <em>Indigofera daleoides</em>, <em>Ozoroa insignis</em>, <em>Spirostachys africana</em>, <em>Schotia brachypetala</em>, <em>Syzygium cordatum</em>, <em>Ximenia caffra</em>, <em>Eucalyptus dives</em>, <em>Eugenia uniflora</em>, <em>Baccharis dracunculifolia</em>, <em>Vernonia polyanthes</em></td>
<td>Mathabe et al., 2006</td>
</tr>
<tr>
<td></td>
<td><em>Naucea latifoli</em></td>
<td>methanol, acetone, ethanol and boiling water extracts</td>
<td><em>V. cholerae</em>, <em>E. coli</em> ATCC 35218, <em>Shigella dysenteriae</em>, <em>Shigella flexneri</em>, <em>Shigella sonnei</em>, <em>Shigella boydii</em></td>
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<tr>
<td>South Africa</td>
<td><em>Elephantorrhiza burkii</em></td>
<td>methanol, acetone, ethanol and boiling water extracts</td>
<td><em>V. cholerae</em>, <em>E. coli</em> ATCC 35218, <em>Shigella dysenteriae</em>, <em>Shigella flexneri</em>, <em>Shigella sonnei</em>, <em>Shigella boydii</em></td>
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<td><em>Eugenia uniflora</em></td>
<td>Essential oil (EO)</td>
<td><em>E. coli</em> O157:H7, <em>Lactobacillus</em> sp.</td>
<td>Broth microdilution with 0.15% agar</td>
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<td><em>Baccharis dracunculifolia</em></td>
<td>Essential oil and methanol extract</td>
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<td>Essential oil and methanol extract</td>
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<tr>
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<td><em>Psidium guajava</em></td>
<td>Essential oil and methanol extract</td>
<td><em>E. coli</em> O157:H7, <em>Lactobacillus</em> sp.</td>
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<tr>
<td>North America</td>
<td><em>Origanum vulgare</em> (Caribbean)</td>
<td>oregano Essential oil</td>
<td><em>E. coli</em> O157:H7</td>
<td>1. Disc diffusion</td>
<td>EO has antibacterial activity against <em>E. coli</em> (max. MBC = 0.08–2.5 µl/ml)</td>
<td>Burt and Reinders, 2003</td>
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<td></td>
<td><em>Thymus vulgaris</em></td>
<td>thyme</td>
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<td>2. Broth microdilution (MIC and MBC)</td>
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<td>North America</td>
<td><em>Caesalpinia pulcherrima</em> (Mexico)</td>
<td>Poinciana Methanol and aqueous extracts</td>
<td><em>E. coli</em> ATCC 25922 (control), clinical isolates of <em>E. coli</em> O157:H7, S. sonnei and S. flexneri</td>
<td>Agar dilution (1, 2, 4 and 8 mg/ml)</td>
<td>These plant extracts were tested at concentration of 8 mg/ml and found to inhibit the growth of tested strains. Methanol extracts have better antimicrobial activities than aqueous solutions</td>
<td>Alanis, <em>et al.</em>, 2005</td>
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<td><em>Chiranthodendron pentadactylon</em></td>
<td>Mexican hand tree</td>
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<td></td>
<td><em>Chrysactinia mexicana</em></td>
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<td></td>
<td><em>Geranium mexicanum</em></td>
<td>(roots and aerial part)</td>
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<td><em>Punica granatum</em></td>
<td>pomegranate</td>
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<tr>
<td>Asia</td>
<td><em>Cinnamomum zeylanicum</em> (Sri Lanka)</td>
<td>cinnamon (bark) water, ethanol and petroleum ether-based extraction, essential oil</td>
<td><em>E. coli</em> O157:H7, <em>E. coli</em> ATCC 25921, <em>E. coli</em> ATCC25922 and <em>E. coli</em> ATCC1105</td>
<td>1. <em>E. coli</em> was cultured with three different concentrations of cinnamon extract. 2. Identification of the antimicrobial compound 3. Broth microdilution(MIC) 4. Disc diffusion</td>
<td>Cinnamon bark has antibacterial activity against <em>E. coli</em>. The antibacterial compound is cinnamic aldehyde.</td>
<td>Muthuswamy, <em>et al.</em>, 2007, Senhaji, <em>et al.</em>, 2007</td>
</tr>
<tr>
<td>Asia</td>
<td><em>Tamarindus indica</em> (Sri Lanka)</td>
<td>Tamarind (leaves, stemp bark, fruit pulp) Ethanol, hot and cold water extracts</td>
<td><em>E. coli</em> from diarrheal stools (7 strains) <em>E. coli</em> ATCC 11775</td>
<td>1. Agar diffusion method 2. Macrodilution method (MIC and MBC)</td>
<td>MBC fruit and bark = 1.25 mg/ml. Only one <em>E. coli</em> strain was resistant to 250 mg/ml tamarind extract. Leaves have the lowest antimicrobial activity.</td>
<td>Nwodo, <em>et al.</em>, 2011</td>
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<td><em>Mangifera indica</em> (Sri Lanka)</td>
<td>Mango (kernel) Ethanol (EtOH) and aqueous extract</td>
<td><em>S. dysenteriae</em></td>
<td>1. Disc diffusion method 2. Agar dilution method</td>
<td>MBC. aq extr. – 0.38 mg/ml, EtOH extr. – 0.19 mg/ml</td>
<td>Rajan <em>et al.</em>, 2011</td>
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<td>Asia</td>
<td><em>Allium sativum</em></td>
<td>Garlic Water extract</td>
<td><em>E. coli</em> O157</td>
<td>1. Growth of <em>E. coli</em> on agar plates containing 1–2% of garlic extract 2. <em>E. coli</em> O/N culture was added to garlic extract and the number of living cells was counted on agar plates</td>
<td>Garlic has antimicrobial activity against <em>E. coli</em> O157. Fresh garlic killed <em>E. coli</em> much faster than 1 year old garlic powder.</td>
<td>Sasaki <em>et al.</em>, 1999</td>
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<tr>
<td>Region</td>
<td>Plant</td>
<td>common name (plant's part)</td>
<td>Method of preparation</td>
<td>Tested enteropathogens</td>
<td>Assay</td>
<td>Result</td>
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<td>2. Disc diffusion</td>
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<td></td>
<td>1. Agar well diffusion method (150 µg/ml)</td>
<td>The lowest activity against tested bacteria with water extract (MIC: 0.156–0.3 mg/ml). With other solvents – MIC: 0.078 mg/ml</td>
</tr>
<tr>
<td></td>
<td><em>Punica granatum</em></td>
<td>(rind and roots)</td>
<td>methanol, acetone, ethanol and boiling water extracts</td>
<td><em>E. coli</em> UP 2566, <em>S. dysenteriae</em> JIA-108, <em>V. cholerae</em>, <em>E. coli</em> ATCC 35218</td>
<td>1. Agar well diffusion method (150 µg/ml)</td>
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<td>2. Broth microdilution (MIC)</td>
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<td></td>
<td><em>Camellia sinensis</em></td>
<td>tea (leaves)</td>
<td>Alcohol extract – dried and brown powder was used</td>
<td>111 bacterial strains were tested including: 15 <em>E. coli</em>, 24 <em>Shigella</em> spp. and 23 <em>V. cholerae</em></td>
<td>Agar dilution method</td>
<td>Tea has antimicrobial activity against studied bacteria. Growth of most <em>E. coli</em> strains was inhibited between 10–20 µg/ml <em>Shigella</em> spp.; 20–30 µg/ml and <em>V. cholerae</em>: 10–30 µg/ml.</td>
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<td></td>
<td><em>Cuminum cyminum</em></td>
<td>Cumin (seeds)</td>
<td>Essential oil</td>
<td><em>E. coli</em> ATCC 35218 and 11 <em>Vibrio</em> spp. strains</td>
<td>1. Disc diffusion</td>
<td>MBC: <em>E. coli</em> – 0.625 mg/ml and <em>Vibrio</em> spp. – 0.31–1.25 mg/ml</td>
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<td>2. Broth microdilution (MIC and MBC)</td>
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<tr>
<td>Asia (India)</td>
<td><em>Azadirachta indica</em></td>
<td>neem (leaves)</td>
<td>methanol extract</td>
<td><em>V. cholerae</em> (NB2 and SG24) – belonged to O1 and O139, PC4, PC9, PC11, PC14 serotypes</td>
<td>1. Disc diffusion</td>
<td>MBC = 10 mg/ml MBC &gt; 5 mg/ml</td>
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<td>2. Broth microdilution (MIC and MBC)</td>
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<tr>
<td></td>
<td><em>Illicium verum</em></td>
<td>Star anise</td>
<td>ethanol and petroleum extracts</td>
<td><em>E. coli</em></td>
<td>Standard agar cup plate method – active compounds were studied</td>
<td>Antimicrobial activity of star anise is mainly due to anethole</td>
</tr>
<tr>
<td>Region</td>
<td>Plant name</td>
<td>common name (plant’s part)</td>
<td>Method of preparation</td>
<td>Tested enteropathogens</td>
<td>Assay</td>
<td>Result</td>
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<tr>
<td>Asia (India)</td>
<td>Ocimum sanctum</td>
<td>Holy Basil (whole plant)</td>
<td>70% alcohol extract</td>
<td><em>E. coli</em> UP 2566, <em>S. dysenteriae</em> IOA-108</td>
<td>Agar well diffusion method (150 mg/ml) – 45 Indian plants were tested</td>
<td>150 mg/ml of plants extracts can inhibit growth of <em>S. dysenteriae</em> and <em>E. coli</em>. Extracts work better against <em>Shigella</em>.</td>
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<td></td>
<td>Morus alba</td>
<td>White mulberry (leaves)</td>
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<td></td>
<td>Hemidesmus indicus</td>
<td>Indian sarsaparilla (roots)</td>
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<td></td>
<td>Eucalyptus sp.</td>
<td>Eucalyptus leaves</td>
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<td></td>
<td>Cassuaria equisetfolia</td>
<td>She-oak (bark and leaves)</td>
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<td></td>
<td>Zizyphus jujuba</td>
<td>Jujube (leaves)</td>
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<td></td>
<td>Syzygium cumini</td>
<td>Jamun (bark and leaves)</td>
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<td></td>
<td>Syzygium aromaticum</td>
<td>Clove (bud and oil)</td>
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<td></td>
<td>Saussurea lappa</td>
<td>(roots)</td>
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<td></td>
<td>Acorus calamus</td>
<td>Sweet flag (rhizome)</td>
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<td></td>
<td>Allium cepa</td>
<td>Onion (leaves)</td>
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<td></td>
<td>Camelia sinensis</td>
<td>Tea (leaves)</td>
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<td>Europe</td>
<td>Vaccinium myrtillus</td>
<td>Blueberry (berries)</td>
<td>Lyophilized berry extract and phenolic compounds</td>
<td><em>E. coli</em> ATCC 11775, <em>E. coli</em> CM871</td>
<td>1. Agar diffusion method 2. Bacteria growth curve <em>Lactobacillus</em> sp. (7 strains) measurement – bacteria grew with 0.5, 1 or 5 mg/ml berry extracts</td>
<td>Finnish berry extracts (1 mg/ml) have antimicrobial activity against <em>E. coli</em> (except Blackcurrant to ATCC 11775). All studied probiotic bacteria are resistant to these extracts</td>
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<td></td>
<td>Rubus idaeus</td>
<td>Raspberry (berries)</td>
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<td></td>
<td>Vaccinium vitisidaca</td>
<td>Lingonberry (berries)</td>
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<td>Ribes nigrum</td>
<td>Blackcurrant (berries)</td>
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<td></td>
<td>Rubus chamaemorus</td>
<td>Cloudberry (berries)</td>
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<td></td>
<td>Vaccinium oxyccocus</td>
<td>Cranberry (berries)</td>
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<td>Hippophae rhamnoides</td>
<td>Sea buckthorn berry (berries)</td>
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<td></td>
<td>Fragaria ananassa</td>
<td>Strawberry (berries)</td>
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<td>Region</td>
<td>Plant name</td>
<td>common name (plant's part)</td>
<td>Method of preparation</td>
<td>Tested enteropathogens</td>
<td>Assay</td>
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<td>Europe</td>
<td><em>Mentha pulegium</em></td>
<td>Squaw Mint (flowering aerial parts)</td>
<td>Essential oil</td>
<td><em>E. coli</em> ATCC 8739, <em>V. cholerae</em> Inaba</td>
<td>1. Disc diffusion</td>
<td>MBC: <em>E. coli</em> – 4 µl/ml and <em>V. cholerae</em> – 1 µl/m</td>
</tr>
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<td><em>Agrimonia eupatoria</em></td>
<td>Agrimony (aerial parts and roots)</td>
<td>Red wine, ethanol (2.5% or 75%) and boiling water extracts (aq)</td>
<td><em>E. coli</em> (UEL 57)</td>
<td>Microdilution method (MIC)</td>
<td>0.2 mg/ml of root aq and EtOH, extract inhibit &gt; 60% <em>E. coli</em> growth</td>
</tr>
<tr>
<td></td>
<td><em>Potentilla reptans</em></td>
<td>Creeping cinquefoil (aerial parts and roots)</td>
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<td><em>Thymus vulgaris</em></td>
<td>Thyme</td>
<td>Essential oil, methanol and aqueous extracts</td>
<td><em>E. coli</em> O157:H7, <em>E. coli</em> ATCC 25922 (control), <em>S. sonnei</em> – 1, 2 and <em>S. flexneri</em> – 1, 2</td>
<td>1. Disc diffusion</td>
<td>Methanol extract has better antimicrobial activity than aqueous. Methanol extract of thyme inhibits 100% growth of all tested strains.</td>
</tr>
<tr>
<td>Europe/Asia</td>
<td><em>Anethum graveolens</em></td>
<td>Dill</td>
<td>Essential oil</td>
<td><em>E. coli</em> O157:H7, <em>Lactobacillus</em> sp.</td>
<td>Broth microdilution with 0.15% agar</td>
<td>EO from coriander leaves have almost two times less antimicrobial activity than seeds (0.4%vol/vol) and is similar to dill. (0.2%vol/vol).</td>
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<td><em>Coriandrum sativum</em></td>
<td>Coriander (leaves and seeds)</td>
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<td><em>Matricaria chamomilla</em></td>
<td>Chamomile</td>
<td>Essential oil and methanol extract</td>
<td>Sixteen <em>E. coli</em> strains isolated from human specimens in the Clinical Hospital of Botucatu Medical School</td>
<td>Agar dilution method</td>
<td>MIC EtOH = 43.4 mg/ml MIC EO = 28.2 mg/ml</td>
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<td><em>Nepeta cataria</em></td>
<td>Catnip (plants at flowering stage)</td>
<td>Methanol extract and essential oil</td>
<td><em>E. coli</em> A1, <em>Shigella</em> spp., <em>E. coli</em> ATCC 43894</td>
<td>1. Disc diffusion</td>
<td>MIC MeOH: 31.25 µg/ml MIC EO: 125 µg/ml</td>
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<td></td>
<td><em>Origanum vulgare</em></td>
<td>Oregano</td>
<td>Essential oil</td>
<td><em>E. coli</em> O157:H7</td>
<td>1. Disc diffusion</td>
<td>EO has antibacterial activity against <em>E. coli</em></td>
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</table>
decoction of guava leaves (Psidium guajava) and unripe bael fruit (Aegle marmelos) was found to have weak bacteriostatic/bacteriocidal properties (Birdi et al., 2010; Brijesh et al., 2009). The study also included tests of adherence/invasion potential of different bacterial species producing enterotoxins to human cells in the presence of plant extract. Because extracts of both plants inhibited the adherence and invasion of bacteria, they could play a positive role in the treatment of bacterial infection by inhibiting the initial colonization of the digestive system by the bacteria. This is a very important but rarely studied phenomena despite the fact that disruption of attachment – the first stage of bacterial infection – is a key property to prevent disease onset. Finding and using plants with this properties could be crucial in case of infections caused by hemolytic strains causing bloody diarrhea that are the most damaging and difficult forms of diarrhea to treat, requiring hospitalization, antibiotic treatment and which cannot currently be alleviated by ORT alone.

The method in which plant extracts are prepared contributes greatly to its efficacy. Prolonged heat treatment might degrade active ingredients present in the initial plant extract or lead to chemical modifications and an increase in the concentration of active ingredients. Examples of different treatment outcomes depending on the extract preparation were described by Rahim et al. and Birdi (Rahim et al., 2010; Birdi et al., 2010). Birdi et al. found that extract of guava (P guajava) leaves were more effective against antibiotic-resistant V. cholerae strains than Birdi, however, Rahim prepared a cold water extract of the guava leaves whereas Birdi heated and reduced the plant extracts (Rahim et al., 2010; Birdi et al., 2010). This suggests that heating may destroy part of the antibacterial properties of the guava leaves although Rahim et al. did not find that heating of their samples reduced their efficacy (Rahim et al., 2010). Variable antimicrobial properties of particular plant extracts can be commonly found in the literature, which stresses the fact that careful attention must be paid to sample collection, storage and preparation, as well as the bacteria strains used in subsequent studies.

The specificity of antimicrobial activities of plant extracts towards pathogenic bacterial strains as compared with benign strains are rarely tested. An example of a study addressing this issue is the test of 26 Mexican plant extracts against non-pathogenic E. coli and the enterotoxigenic E. coli O157:H7, amongst others (Alánis et al., 2005). The aqueous extracts used in these studies showed a much better specificity for the pathogenic E. coli O157:H7 strain over a non-pathogenic E. coli strain when compared to methanolic extracts. Carica papaya (papaya), Ocimum basilicum (basil), Matricaria chamomilla (chamomile) and Thymus vulgaris (thyme) are commonly known plants reported with such antimicrobial specificity. Patient recovery rates and reestablishing gut flora, which can take several weeks following hospitalization, are an important consideration with respect to diarrhea. The retention of any beneficial bacteria in the digestive system by the selective action of plant extracts has much to be recommended.

Organic solvents, chiefly ethanol and methanol, are often used to obtain active ingredients from plant extracts but using them in the treatment of patients with acute diarrhea is highly questionable. A refluxed ethanolic extract of cinnamon (Cinnamomum zeylanicum) inhibited the growth of Listeria innocua and E. coli O157:H7 but an aqueous extract was ineffective (Muthuswamy, Rupasinghe and Stratton, 2007). The 50-fold dilution of the ethanolic extracts means that the final solution still contains 2% ethanol, which is unsuitable for the treatment of dehydrated patients, particularly children. Senhaji found that the essential oil of the same plant, obtained without organic solvents, was effective against E. coli O157:H7 (Senhaji, Faid and Kalalou, 2007). In this case pure essential oil could be a second choice for studies, after simple water extracts.

Plant metabolite concentrations often vary during the growth cycle and in different parts of the plant so a consistent level of therapeutic molecules is an important consideration. For Nepeta cataria (catnip), the antibacterial properties of essential oils distilled at three different stages of plant growth were tested (Zomorodian et al., 2012). In this case, consistent results were obtained across the growth stages which uphold specificity for S. aureus and Shigella sp. over E. coli. It is worth noting that the essential oil content of catnip tea will be much lower than the levels of essential oils reported to have an antibacterial effect and also that catnip tea is not recommended for women during pregnancy or lactation (Ernst, 2002).

A range of European berries (blueberry, raspberry, lingonberry, blackcurrant, strawberry, cloud berry, sea buckthorn berry and cranberry) were found to be more active against E. coli and other pathogenic bacteria compared to Lactobacillus sp. (Puupponen-Pimiä et al., 2001). However, the samples were first extracted with 70% acetone and processed to remove sugars. It is not possible to estimate the amount of fruit required providing equivalent levels of active material and if a low concentration of fruit extract could replace part of the sugar component in ORT. Positive data for raspberry fruit and cordial at 10% dilution has also been reported (Ryan, Wilkinson and Cavanagh, 2001). While neat fruit juices are generally not recommended for the treatment of diarrhea, these studies support the potential inclusion of natural, rather than artificial, fruit flavors in ORT formulas.

Publications naturally report positive antimicrobial properties of plant extracts but it is not always easy to
Principal ingredient of thyme oil, thymol, is used in mouthwashes as an antibacterial and antifungal agent but there are no safe recommendations about its use in food. Oregano oil is generally recognized as safe by the FDA at a consumption level of 200 mg a day (Food Listing of Food Additive Status Part II. US Food and Drug Administration Web Site. http://www.fda.gov/ Food/FoodIngredientsPackaging/FoodAdditives/ ucm191033.htm#ftnO). The bacteriostatic and bactericidal properties of oregano varied with the presence of other added ingredients (agar and soy lecithin).

Garlic is considered beneficial for a wide range of medical problems. Included as a 1% additive to media, garlic was active against several bacteria, including E. coli O157:H7 (Sasaki et al., 1999). Fresh garlic extract and allicin, the principal compound in garlic suspected of carrying antimicrobial properties, had equal or better activity than five tested antibiotics against a range of antibiotic resistant strains of Shigella sp., enterotoxigenic E. coli and V. cholerae. (Ahsan et al., 1996). Allicin is an unstable molecule and the effectiveness of garlic is highly dependent on the source of the plant material, method of preparation, as well as the ages of the plant material and extract. Allicin is not the only active component of garlic, Politi et al. identified a polysaccharide component isolated from garlic that binds to the B-subunit of cholera toxin to confer antitoxin properties (Politi et al. 2006).

4. Avenues for future research

The majority of published works in the field focus on the antimicrobial properties of plant extracts. Few studies test the specificity of plant extracts for infectious over benign bacterial strains. The toxicity of plant extracts to humans should also be considered, particularly when highly concentrated fractions are used, or parts of the plant that are not normally ingested. Pharmaceutical practice focuses on developing one drug or treatment to target a specific and weak point in the disease mechanism. In principal, a mixture of plant extracts may present a synergistic effect against several stages of the infection cycle, specificities against a wide range of bacteria and overall lead to obtaining a more effective remedy for diarrhea.

The search for beneficial plant extracts would benefit from multi-disciplinary, collaborative, large-scale screening approaches. The first task is to select plant materials that are generally considered safe for human consumption (including children and pregnant/lactating women) as based on literature searches. Plant material should be collected at different growth stages and from different varieties of plant. Screening of fresh, stored and dried plant materials should be performed. Plant extracts should be prepared with cold water, hot...
water and boiling/refluxing extractions – toxic solvents should be avoided. The long-term stability of the extracts in solution and/or as freeze-dried powders should be compared.

Antimicrobial screens should be based around well-established protocols to measure bacteriostatic and bactericidal properties of the plant extracts. However, it is important to test a range of enterotoxic and benign strains of bacteria in order to obtain ideas on the specificity of plant extracts. Advanced protocols based on mammalian cell cultures to test the anti-adhesion and anti-invasion properties of plant extracts on bacterial cell cultures are critical to the discovery of plant extracts that can counter hemolytic strains of enterotoxigenic bacteria. Protocols to measure the downregulation of toxin production (rtPCR) and toxin secretion by plant extracts are worth carrying out to ascertain if plant extracts are capable of slowing diarrheal episodes.

Returning to sample selection, combinations of optimal plant extracts should be tested in combination to ensure that their full activity is maintained or synergy obtained. The final aim is to have ready-to-use ORT in solution and sachets of dry ORT to be dissolved in clean water. These products fulfill the aim of treating diarrhea but prevention and control of the disease might be aided with sachets of dried plant extract (without added salt and sugar) or teabags filled with mixed plant materials.

The strategy outlined above means a change from the current approach of validating plant extracts on an individual or regional basis towards a more multidisciplinary, collaborative effort. We have recently instigated a project targeted towards AB5-producing bacteria, establishing a range of protocols in three different labs. Our antimicrobial data corroborate existing published data for a number of hot water plant extracts and reveal new, include toxicity studies on human cell lines and a number of anti-toxin properties. However, we still do not employ a full range of protocols or have instigated animal or human trials to test our suggested mixes of plant extracts. Furthermore, the AB5 family of enterotoxins represents just one group of enterotoxic pathogens that can be studied using the same set of protocols.

5. Conclusion

It is not possible to compare datasets for a particular plant due to different plant sources, extract preparations and microbial screens. However, the wide variability indicates that these points are very important being very important in determining if a particular plant extract will be effective or not. A plant extract, or more likely a mixture of plant extracts, that provide a wide spectrum of antimicrobial and anti-toxin properties would provide a powerful boost to ORT formulations. It is important in such a case that a suitable antimicrobial screen is used to assay the plants before distribution or sale. A second point is that several screens are required to test plant effects against different aspects of microbial colonization, toxin release and toxicity. It is this point that is largely overlooked in favor of traditional, plate-based antimicrobial screens. Future studies would benefit from large-scale collaborative screening with an aim of improving ORT.

Acknowledgement
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Literature


Quantitative Expression of Candida albicans Aspartyl Proteinase Genes SAP7, SAP8, SAP9, SAP10 in Human Serum in vitro

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A b s t r a c t

The different members of the secreted aspartyl proteinase (Sap) family of the human pathogenic yeast Candida albicans are proposed to play different roles during infection and are differentially expressed at various body sites. In recent reports, expression analysis has focused on the genes SAP1-6, while the expression pattern of SAP7-10 was less well studied. We analyzed the SAP7–SAP10 expression profile of C. albicans under human serum influence that may be elucidated in the course of blood infection in humans and how this in vitro expression profile is associated with hyphal formation. The phenotypes of strains were examined under scanning electron microscopy. Quantitative RT-PCR under human serum influence that may be elucidated in the course of blood infection in humans and how this

K e y w o r d s: Candida albicans, aspartic proteinases expression, morphogenesis, blood human serum

Introduction

Candida albicans possesses a number of virulence attributes and among them widely studied are aspartyl proteinases (Saps) that can digest epithelial cell surface components and thereby provide an entrance into or between host cells (Dalle et al., 2010). Although plenty of data dealing with the Saps involved in the infection process have been extensively studied (Naglik et al., 2008; Rehaume et al., 2008; Schelenz, 2008) the comparative analysis of Saps expression in morphologies induced under human serum influence at human body temperature has not been described in depth. Given the role of SAP7, SAP8 and SAP9-10 in the growth of C. albicans in serum (Lermann and Morschhäuser, 2008), we asked whether those isoenzymes are expressed in C. albicans hyphal forms induced under human serum influence in vitro. In addition, we analyzed whether any differences exist in the expression level between these subfamilies. In contrast to all other members of the Sap family, the Sap9–10 proteases monitored under in vitro and in vivo conditions are independent of pH and morphotype (Hornbach et al., 2009; Schild et al., 2011). Furthermore, according to Schild et al. (2011) it seems apparent that the host associated lifestyle of C. albicans requires regulatory proteolytic digestion at different niches with different pH values. Moreover, the latter authors demonstrated that Sap9 and Sap10 influence on cell wall functions by proteolytic cleavage of cell wall proteins which are significant for morphogenesis (Schild et al., 2011). Thus, in the current study SAP9 and SAP10 expression analysis was undertaken in hyphal forms under serum influence at pH 7.1–7.4. An earlier study (Naglik et al., 2003) showed that SAP7 was found to be induced in response to infection of reconstituted human vaginal epithelium model as well as in isolates from patients. Furthermore, Taylor et al. (2005) observed that induction of SAP7 correlates with virulence in an intravenous infection model of candidiasis in mice. Many differences in the
SAP gene expression profiles observed in various studies remain to be explained and more thoroughly characterized. Therefore, the aim of the study was to determine the relative levels of SAP7, SAP8, SAP9 and SAP10 transcripts in *C. albicans* strains *in vitro* at neutral pH at 18 h-growth in human blood serum (an artificial model of bloodstream infections). That may provide evidence for a role of these proteinases in *C. albicans* survival and escape of disseminated infection. Moreover, we determined whether any correlation might exist between overexpression of the SAP7-10 genes and true hyphae formation.

**Experimental**

**Materials and Methods**

**Strains and growth conditions.** The *Candida* strains used in this study are listed in Table I. The stock culture of examined strains was stored on ceramic beads (Microbank™, Pro-Lab Diagnostics, Canada) at –70°C. Routine culturing of strains for growth in YEPD medium as well as for morphogenesis development in human serum was conducted as described previously (Staniszewska et al., 2012). The usefulness of fresh human serum in order to mimic blood serum infections and to investigate the gene expression pattern of *C. albicans* during such infections incubated under semi-aerobic conditions at 37°C was described by Fradin et al. (2003; 2005).

**Quantitative RT-PCR.** Total RNA was isolated from cells according to the protocol by Amberg et al. (2005). RNA concentrations were determined by measuring absorbance at 260 nm (Nano Drop 2000, Thermo SCIENTIFIC, USA). First-strand cDNAs were synthesized from total RNA, using two-step qRT-PCR analysis (Sigma, USA) and following the recommendations of the manufacturer. Real-time RT-PCR was used to determine the quantitative levels of SAP7-10 mRNA transcripts in RNA samples using Rotor Gene 6000 (RCorbett, Qiagen, Germany). Quantitative reverse transcription-PCR assay was performed following the protocol described previously by Naglik et al. (2008) using QuantiTect Probe PCR Kit (Qiagen, Germany) according to the manufacturer’s instructions. TaqMan primer and probe (5′ FAM, 3′ TAMRA) sets were used as described previously Naglik et al. (2008). The 2^−ΔΔC_T method was used to analyze the relative changes in gene expression from quantitative RT-PCR experiment (Livak and Schmittgen, 2001). The C_t values were provided from RT-PCR instrumentation and were imported into a spreadsheet Microsoft Excel 2010. The data were analyzed using Eq. (Livak and Schmittgen, 2001), where ΔC_T = Avg. sap C_T – Avg. β-actin C_T and ΔΔC_T = ΔCT – ΔCT parental strain.

**Scanning electron microscopy study.** To examine cell morphology, samples were prepared as described previously by Oliviera et al. (2010). Briefly, morphotypes of each strain were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 18 h at a low temperature (5–6°C). Then, the samples were carefully washed with 0.1 M phosphate buffer (pH 7.2). Post-fixation was carried out for 2 h at room temperature with 2% osmium tetroxide. Initial dehydration was accomplished by placing specimens in the following series of ethanol gradients: 50% and 70% (two times for 10 min), 95% (two times for 5 min) and 100% (two times for 1 min), respectively. Then, samples were dehydrated with acetone (two times for 30 sec.) until dried by the critical point method in liquid CO_2. Subsequently, the specimens of the wild type strain were coated with gold in a vacuum evaporator and examined with a scanning electron microscope (SEM Quanta-200, FEI, Czech Republic). In the case of the mutant strains, after post-fixation morphotypes were dehydrated two times for 10 min with graded ethanol (50% and 75%) followed by dehydration with acetone (two times for 30 sec). The dried specimens were coated with osmium tetroxide and observation was done under scanning electron microscope Hitachi S-5500 (Japan). Images were assembled with Photoshop (Adobe Photoshop CS3 Extended). Cell dimensions were determined by using bars.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Parent</th>
<th>Genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>SC5314</td>
<td>Wild strain</td>
<td></td>
<td>Gillum et al. (1984)</td>
</tr>
<tr>
<td>83</td>
<td>Clinical isolate</td>
<td></td>
<td>Staniszewska (2009)</td>
</tr>
<tr>
<td>CAF2-1</td>
<td>SC5314</td>
<td>Δura3::imm434/URA3</td>
<td>Fonzi and Irwin (1993)</td>
</tr>
<tr>
<td>Δsap9</td>
<td>CAI4[pCIp10]</td>
<td>Δsap9::hisG/Δsap9::hisG + pCIp10 (Integration)</td>
<td>Schild et al. (2011)</td>
</tr>
<tr>
<td>Δsap9/10</td>
<td>CAI4[pCIp10]</td>
<td>Δsap9::hisG/Δsap10::hisG Δsap9::hisG + pCIp10 (Integration)</td>
<td>Schild et al. (2011)</td>
</tr>
<tr>
<td>Δsap10</td>
<td>CAI4[pCIp10]</td>
<td>Δsap10::hisG/Δsap10::hisG + pCIp10 (Integration)</td>
<td>Schild et al. (2011)</td>
</tr>
</tbody>
</table>
Expression of C. albicans aspartyl proteinase in human serum

Results

Quality control and quantitative expression of SAP7–SAP10 genes in human blood serum. SAP7, SAP8, SAP9–10 and ACT1 TaqMan primer/probe sets were calibrated and they demonstrated similar efficiency in titration experiments using C. albicans SC5314 genomic DNA (9.7–52.3 ng) in serial log10 dilutions (data not shown). The expression levels of SAP7–SAP10 genes were monitored after a 18 h period after inoculation of human serum with the C. albicans strains (Fig. 1). All SAP genes tested were detected in human serum, albeit at very low levels, except SAP7, which was expressed at relatively high levels compared to the remaining genes tested (Table II). Contrariwise, the wild type strain 83 and CAF2-1 showed slightly enhanced SAP10 expression (at least 1.2- and 2.3-fold) compared to SAP7 respectively. We therefore examined the expression of SAP7–10 genes under serum influence by comparing the expression profile among particular mutants. Considering SAP7, in the double mutant Δsap9/Δsap10 the expression level of SAP7 was significantly higher (20-fold compared to Δsap9 or 8.4-fold compared to Δsap10) and so was that of SAP8 (5.0- or 3.3-fold for Δsap9 and Δsap10 respectively). As a result of the comparison of the expression pattern of wild type strains it was shown that in the reference strain SC5314 SAP7 was expressed 1.5-fold compared to CAI4. Contrariwise, the wild type CAI4 displayed around 1.6-fold higher expression of SAP9 than strain 83. We demonstrated that CAF2-1 has a 2.3-fold change of SAP10 gene expression compared with either CAI4 or strain 83. Contrariwise, the wild type SC5314 showed 5-fold lower expression (compared to strain 83 and CAI4) and 11.5-fold lower expression than CAF2-1. Comparison of the expression patterns of the protease mutants and the wild types demonstrated high diversity. The expression levels of SAP7, SAP8 and SAP10 in the wild type CAF2-1 were around 3.3- and 5.0- and 46-fold higher than in mutant the Δsap9 respectively. Moreover, in the mutant Δsap9 SAP10 was underexpressed 20-fold vs the parental strain CAI4. In view of SAP10, the mutant Δsap9 displayed expression from 4.0- to 21-fold lower than the wild types. Similarly, deletion of SAP9 or SAP10 appeared to have a minor influence on expression of either SAP8 or SAP9 (comparable to the wild types). Thus, Δsap10 as well as SC5314 displayed comparable expression of SAP9 (2Δ∆CT = 0.3 or 0.2 respectively).

When comparing the three genes (SAP7 vs SAP8 vs SAP9) in the mutant Δsap10 at 18 h-growth in human

<table>
<thead>
<tr>
<th>Gene</th>
<th>Fold mRNA expressiona in C. albicans strains</th>
<th>Fold mRNA expression relative to ACT1 and normalized over the value of the wild type strains expression</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>CAI4</td>
<td>SC5314</td>
</tr>
<tr>
<td>SAP7</td>
<td>1.4</td>
<td>2.1</td>
</tr>
<tr>
<td>SAP8</td>
<td>0.2</td>
<td>0.3</td>
</tr>
<tr>
<td>SAP9</td>
<td>1.0b</td>
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</tr>
<tr>
<td>SAP10</td>
<td>1.0b</td>
<td>0.2</td>
</tr>
</tbody>
</table>

a – Values are average of triplicate readings; b – Parental strain; ND – not determined

Fig. 1. Expression of the SAP genes.
The expression of SAP7, SAP8, SAP9 and SAP10 was quantified by qRT-PCR from cells in stationary growth phase in human serum at 37°C. ACT1 transcript was used as internal control and the multifold increase over the value of the wild type at 37°C is shown for each strain.
serum, one can see a clear trend in underexpression of these genes compared to the wild types, with the exception of SAP8 upregulation (1.5-fold) compared to CAI4. Furthermore, SAP7 and SAP8 were expressed 1.4-fold and 3.3-fold higher in the wild type CAF2-1 compared to Δsap10 respectively. Likewise, in the parental strain CAI4 SAP9 displayed 5.0-fold overexpression vs Δsap10. The double mutant Δsap9/Δsap10 showed an increased expression of SAP7 (2.8-fold compared to the wild type SC5314). Furthermore, Fig. 1 and Table II showed that SAP7 was clearly the most highly expressed gene at around 5.9-fold (with reference to CAI4-1) in the Δsap9/Δsap10 mutant. Consequently, SAP8 displayed a significant overexpression in the Δsap9/Δsap10 mutant compared to the wild types SC5314 and CAI4 (in the range 3.3 and 5.0-fold, respectively). While in the double mutant Δsap9/Δsap10 the expression level of SAP8 was comparable to CAI2-1.

Hypha-forming capability in *Candida albicans* strains. In order to characterize the phenotype of Δsap, we examined the ability to undergo the dimorphic transition. For this purpose, cells were grown in liquid undiluted human serum and incubated at 37°C, cell morphology was then observed microscopically after 18-h incubation. To test this possibility, we examined which genes could prevent from the morphogenesis defect of Δsap9/Δsap10 in human serum. As shown in Fig. 2, the mutants Δsap9 and Δsap9/Δsap10 exhibited decreased ability to filament compared to the wild type cells. Although, the latter mutants are able to form filaments, they fail to filament abundantly in these preferable conditions probably because the absence of SAP9 represses the dimorphic transition in *C. albicans*. Contrariwise, the Δsap10 mutant formed true hyphae when grown under these conditions as revealed by microscopic examination. The deletion of genes SAP8 (data not shown) and SAP10 had no effect on morphogenesis process.

Discussion

Our results demonstrated that from the tested SAP7 to SAP10, only SAP7 mRNA level is significantly altered at the later stage of bloodstream infections in different genetic backgrounds (the wild types and the Δsap mutants). Comparing the expression of SAP9 and SAP10, our assay suggested that the single mutants lacking either of these proteins do not show a significant expression increase of the other. We found that the expression levels of SAP7-10 were downregulated in all the single mutants compared with the wild types. One possible explanation for this observation may be that the single mutants have a reduced ability to survive in blood serum. Contrariwise, the double mutant Δsap9/Δsap10 displayed overexpression of SAP7 and SAP8 compared to the wild types. These results support the notion that compensatory upregulation of SAP7 and SAP8 in the Δsap9/Δsap10 mutant occurred. However, the mechanism underlying how and when SAP9 and SAP10 regulate other genes as well as each other remains to be elucidated. Interestingly, lack of Sap10 had only a minor influence on SAP9 expression (4-fold higher) compared to the expression level of SAP10 in Δsap9. Recently, Schild et al. (2011) showed that although Sap9 and Sap10 exhibit near neutral pH optimum of proteolytic activity they only partially substitute each other in these conditions. In our study a lower expression of SAP10 may be related to a more limited number of potential substrates (Schild et al., 2011).

In the study, the mutants lacking these proteins showed dissimilar phenotypes of Δsap9 or Δsap10, such as altered morphogenesis under human serum influence. Moreover, our data supported the results by Naglik et al. (2004) that Sap9 is probably a key proteinase that does not only promote cell wall integrity, general cell growth and fitness at mucosal surface, but as our data implied is dependent on the morphology under human serum influence. Thus, our study supports the findings of Schild et al., (2011) and in view of our data it is highly probable that Sap9 is involved in polarized growth and morphogenesis in *C. albicans* under human serum influence at pH 7.1–7.4. Previously, quantification of cellular forms of the wild type strain (Staniszewska et al., 2011 and 2012) showed that incubation in human serum at 37°C for 18 h resulted in transformation of almost 100% of all the observed cells to pure true hyphal forms. However, our present data demonstrated that deletion of SAP9 substantially reduces true hyphae formation compared with the wild type strains *i.e.*, strain 83, SC5314 as well as Δsap10 under these conditions (Fig. 2). Thus, SAP9 expression may be linked to hyphal morphology even if the pleomorphism is regulated by other factors. Moreover, it was shown that incubation in undiluted serum at human body temperature did not prevent the defective hyphal growth of the mutants Δsap9 and Δsap9/Δsap10 (Fig. 2), indicating that Sap9 may be involved in the loss of cell polarity in these mutants under hyphae-forming conditions. Moreover, we showed that SAP7 and SAP8 expression could not prevent the morphogenesis defect of either Δsap9 or Δsap9/Δsap10 in undiluted human serum and expression of these genes does not induce hyphae formation. Finally, our data imply that Sap7, Sap8 and Sap10 play a minor role in morphogenesis and these genes do not compensate the SAP9 disruption in hyphae formation.

Studies investigating the production of SAP mRNA (Hube et al., 1994; Taylor et al., 2005) and the virulence of SAP knockout strains (Lermann and Morschhäuser, 2008) showed that induction of SAP expression is a niche-specific phenomenon. Therefore it was necessary in our study to discover which of the tested protein-
ases are expressed at higher level during artificial blood infection. We observed that human serum favours \textit{SAP7} expression. One reason for this unexpected result may be found in the fact that \textit{SAP7} plays a role in blood dissemination followed by interaction with human endothelial cells. These results demonstrated that \textit{SAP7} is required for the growth of \textit{C. albicans} clinical isolates in human serum as the sole nitrogen source and Sap7 is normally expressed in the wild type strains and over-expressed when the remaining genes are expressed at a very low level. It was likely in our study that when \textit{C. albicans} cells have to adapt and survive within the blood environment they upregulate \textit{SAP7} expression, while other genes important for growth and viability in serum are downregulated (Fradin \textit{et al.}, 2003; Hube \textit{et al.}, 1994). We therefore concluded that there is little

Fig. 2. Characteristics of \textit{Candida albicans} strains' phenotypes grown in undiluted human serum at 37°C for 18 h under static conditions in plastic Eppendorf vials.

(A) Micrograph showing true hyphal phenotype of the \textit{C. albicans} clinical strain 83. Note the dominant filament morphotypes (arrows). (B) Clumps of morphological forms of strain SC5314 can be seen. Note the germinating blastoconidial cell, indicated by an arrow and true hyphal morphologies (open arrows). (C, D) The \textit{\textDelta sap9} mutant exhibits budding phenotype and failure of hyphal development. Fine details of blastoconidia are discernible. (C) Oval blastoconidial mother cell (arrow) with a bud (open arrow) can be seen. Scars (arrowheads) localized polarly are observed. (D) Blastoconidial cells morphology and cellular budding (arrow) typical of strain the \textit{\textDelta sap9} are visible. Mutant causes filamentous growth only in a small percentage of yeast cells. Note singular hyphal morphology (open arrow). (E) The \textit{\textDelta sap10} mutant. Clumps of germinating blastoconidia can be seen (arrow). Hyphal induction has occurred, note long true hyphal cells indicated by arrow heads. (F) The \textit{\textDelta sap9/\textDelta sap10} mutant phenotype. Yeast cells defective in cellular filamentation process are observed. Fine details of blastoconidia are discernible. Oval blastoconidial cell (arrow) and a new bud (open arrow) can be seen. Scars (s) localized polarly are observed.
correlation between the expression of the remaining SAP genes tested and the role of Sap proteins during blood infection. Furthermore, our findings were in agreement with the view that protein production is reduced in the later stage of blood infections (Fradin et al., 2003). Thus, in our opinion future experiments using patients’ blood samples should bring us closer to identifying the SAP genes and proteins that are directly involved in candidaemia in humans. Furthermore, a gradual observation of SAP expression during blood infection ought to be conducted which may show whether SAP7 is induced as a consequence of infection. Taylor et al., (2005) utilizing the ∆sap7 mutant showed a role of proteinase Sap7 in the intravenous model of systemic candidiasis in mice which was in agreement with our in vitro blood infection model in humans. Furthermore, our study was a successful attempt in finding conditions which induce SAP7 in vitro while it was discussed (Taylor et al., 2005) that an in vitro induction of SAP7 mRNA level has not yet been detected. In conclusion, these data provided the first in vitro evidence of SAP7-10 expression in C. albicans strains under human serum influence. In this paper, due to the strong induction of SAP7, we hypothesize that it is essential for C. albicans survival and help the cells to escape from bloodstream. Thus, SAP7 may help the fungus to cause systemic infections. We also report SAP9 expression in serum, which was associated with hyphal invasive growth at the site of infection.

Acknowledgement

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Literature

The Effect of Oleanolic and Ursolic Acids on the Hemolytic Properties and Biofilm Formation of *Listeria monocytogenes*

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**Abstract**

Oleanolic acid and ursolic acid are pentacyclic triterpenoids isolated from a variety of medicinal plants, which have antibacterial activity. *Listeria monocytogenes* is a Gram-positive facultative pathogen, being the causative agent of listeriosis. The present study was carried out to evaluate the *in vitro* effect of sub-inhibitory concentrations of both triterpene acids on the pathogenicity determinants of *L. monocytogenes*: their hemolytic activity and biofilm forming ability. Oleanolic and ursolic acids inhibited listeriolysin O activity without influencing toxin secretion. Biofilm formation, and the viability of *L. monocytogenes* cells in biofilms was diminished by both compounds. Thus, both acids affected *L. monocytogenes* virulence. It was also demonstrated that oleanolic acid bound to the peptidoglycan of *L. monocytogenes* and this interaction was influenced by teichoic acids.

**Key words:** *Listeria monocytogenes*, biofilm, listeriolysin O, oleanolic acid, ursolic acid

**Introduction**

Compounds of therapeutic value extracted from medicinal plants are mostly secondary metabolites (Cowan, 1999). Oleanolic acid (OA) and ursolic acid (UA) are representatives of the pentacyclic triterpenoids whose structures are based on the isoprene moiety. Both compounds exhibit several pharmacological activities. The hepatoprotective, anti-inflammatory, antioxidant and anticancer activities of OA and UA are well documented (Ikeda et al., 2008; Pollier and Goossens, 2012). OA, UA and their derivatives also possess antibacterial activity, primarily against Gram-positive bacteria, including multidrug-resistant strains (Fontanay et al., 2008; Wolska et al., 2010). However, the broad application of OA/UA is currently restricted because their mechanism of action is still poorly understood and the side-effects on eukaryotic cells have not been fully characterized.

A relatively small number of studies have investigated the cellular targets and functions affected by OA/UA. Ren and coworkers (2005) showed that UA caused differential gene expression in *Escherichia coli* and substantially inhibited biofilm formation by *E. coli*, *Pseudomonas aeruginosa* and *Vibrio harveyi*. Subsequently, Chen and coworkers (2009) demonstrated the ability of the oleane-type triterpenoid, glycyrrhizin, to inhibit the diarrhea-inducing activity of the heat-labile enterotoxin produced by ETEC (*enterotoxigonic *E. coli*). The interaction between OA/UA and several other antibacterial agents – mainly conventional antibiotics – has been also reported (Ge et al., 2010). Of particular interest is their synergistic action with β-lactam antibiotics against *Staphylococcus aureus* and *Staphylococcus epidermidis* (Kurek et al., 2012) and the ability to induce stress response (Grudniak et al., 2011).

The Gram-positive pathogen *Listeria monocytogenes* is the causative agent of listeriosis, a disease that has increased markedly in Europe in recent years (Allerberger and Wagner, 2010). Listeriolysin O (LLO) is considered as a major virulence determinant of *Listeria* (Kayal and Charbit, 2006; Schnupf and Portnoy, 2007). Recent studies showed new roles for intracellular LLO, such as control of autophagy upon vacuolar escape, and revealed the extracellular activities of LLO, for example induction of bacterial entry into the cells and modulation of immune activity (Hamon et al., 2012). *L. monocytogenes* forms biofilms with a structure that varies greatly from one strain to another. Bacteria living in biofilms are less susceptible than planktonic cells to antimicrobial...
agents, especially antibiotics (Renier et al., 2011). The factors responsible for this enhanced resistance include restricted penetration of antimicrobials into the biofilm structure, decreased growth rate and the expression of possible resistance genes (Lewis, 2001). For this reason, the ability to grow in a biofilm is known to enhance the pathogenicity of certain bacterial species.

It was previously proved that the cell wall constitutes a cellular target of OA/UA activity against the facultative pathogen Listeria monocytogenes. Both these pentacyclic triterpenoids affected morphology and enhanced autolysis of bacterial cells, influenced autolysis of the isolated cell wall, inhibited peptidoglycan turnover and quantitatively changed the profile of muropeptides obtained after the digestion of peptidoglycan with mutanolysin (Kurek et al., 2010). So far there is no available information on the ability of peptidoglycan to bind either OA or UA.

The aim of the present study was to gain some insight into the effect of OA/UA on L. monocytogenes virulence factors. The influence of these compounds on the secretion and activity of listeriolysin O, biofilm formation and L. monocytogenes viability in biofilms was examined. As peptidoglycan is involved in L. monocytogenes biofilm formation, the ability of UA/OA to bind this compound was also studied.

Experimental

Materials and Methods

Bacterial strain, medium and reagents. Listeria monocytogenes (PCM 1291) was obtained from the Polish Culture Collection, Wrocław, Poland. The bacteria were grown in tryptic soy broth with yeast extract – TSYEB at 37°C. OA, UA and all other reagents were purchased from Sigma. OA containing a tritium-labeled hydroxyl group (3H-OA, 1 mg mL⁻¹, activity 5.1 GBq mmol⁻¹) was provided by the Department of Plant Biochemistry, Faculty of Biology, University of Warsaw.

Measurement of the hemolytic activity of L. monocytogenes. An overnight culture of L. monocytogenes was grown in TSYEB medium with constant shaking. After dilution to attain an A₆₀₀ of 0.1, the cultures were incubated further to an A₆₀₀ of 0.5. Cells were harvested by centrifugation, and proteins from the supernatants were precipitated and concentrated using 10% trichloroacetic acid (TCA). The protein concentration in each sample was estimated using the bicinchoninic acid assay (Brown et al., 1989). Samples containing 5 μg of precipitated protein were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) and the resolved protein bands were transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were incubated with rabbit anti-LLO polyclonal antibody (Abcam, UK, 1:1500 dilution) for 1 h, washed, then incubated with goat anti-rabbit IgG – alkaline phosphatase conjugate, washed again and finally developed using 5-bromo-4-chloro-3-indolyl phosphate and nitro-blue tetrazolium (BCIP/NBT) in carbonate buffer as the chromogenic substrate. Images of the membranes were captured and analyzed using Image Master VDS (Fujifilm) and Image Master 1D Elite version 301 programs (NonLinear Dynamics, UK).

Quantitative determination of biofilm formation and measurement of cell viability in biofilms. Bacterial biofilms were developed in wells of polystyrene microtiter plates containing 200 μL samples of culture diluted in TSYEB medium supplemented with 0.45% glucose and various amounts of the tested compounds. The lowest concentration of a compound that inhibited biofilm growth, as determined by crystal violet staining, was taken as the minimal biofilm inhibitory concentration (MBIC) (Smith et al., 2008). To determine the amount of biofilm in the wells of polystyrene microtiter plates, those formed after 24 h in the presence of OA, UA or with control samples were stained with crystal violet and, after brief washing with 0.85% NaCl, ethanol was added to each well to solubilize the crystal violet and the dye concentration in this wash was estimated by measuring the A₅₇₀ with a microtiter plate reader (Sunrise, Tecan, Switzerland). To measure cell viability in biofilms, the 3-(4,5-diethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) staining method was employed (Kairo et al., 1999) with minor modifications (Walencka et al., 2005). The intensity of the violet color of the resulting formazan was determined by measuring the A₅₇₀ with a microtiter plate reader.

Measurement of the ability of OA to bind peptidoglycan. Peptidoglycan was isolated from L. monocytogenes.
genes cells following the procedure described by Korsak and coworkers (2005). The final peptidoglycan preparations were divided into two parts: one part was lyophilized and the second was treated with 5% TCA in order to remove teichoic acids. The prepared peptidoglycan samples were suspended in distilled water (1 mg mL\(^{-1}\)) and amounts of 100 µg were incubated with \(^3\)H-OA (0.05, 0.25, 0.5 or 1 µg) for 30 min at room temperature. The binding reactions were terminated by adding a 10-fold excess of unlabeled OA followed by 5 min incubation. The samples were centrifuged (16,000 g, 10 min), then the resulting pellets were washed with distilled water and analyzed using a scintillation counter. In the negative controls, the cell wall fragments in the binding reaction were replaced by water.

**Statistical analysis.** Data are shown as means of at least three experiments ± SD. Statistical significance of the differences between experimental groups was calculated using two-tailed unpaired Student’s test. \(p\) value < 0.05 was considered statistically significant.

**Results and Discussion**

The hemolytic activity of the supernatants of *L. monocytogenes* cultures treated with 0.5× or 0.75× minimal inhibitory concentration (MIC) of OA/UA and an untreated control culture was determined. MIC values of both compounds were 8 g mL\(^{-1}\) as determined previously (Kurek *et al*., 2012). Both triterpene acids inhibited LLO hemolytic activity. Compared with the control, the inhibition observed in cultures treated with 0.75 × MIC of OA or UA amounted to 2.5-fold and 2.8-fold, respectively (Fig. 1). As it was demonstrated that triterpenoids are able to form complexes with sterols (Osborn, 1996) and cholesterol are present in erythrocyte membrane, the influence of OA/UA on erythrocytes in the absence of LLO was determined in the control experiments. It was shown that OA and UA in concentration 0.015 × MIC, i.e. equivalent to 0.75 × MIC present in LLO-containing supernatants (supernatants were diluted 50 × in hemolytic activity assay, see Material and Methods) did not cause the lysis of erythrocytes. Analysis of SDS-PAGE followed by Western blotting demonstrated that the level of LLO in the culture supernatants was not diminished by treatment with either OA or UA (Fig. 2). The differences between densitometric measurement of peak areas did not exceed 10%. The synthesis of LLO was also not influenced by either of these compounds, since the amount of this cytolsin in whole cell lysates was unchanged by OA/UA treatment (data not shown). LLO is translated as a precursor polypeptide of 529 residues and its N-terminal signal sequence is cleaved after protein secretion (Mengaud *et al*., 1988). The results of the present study indicate that OA and UA do not interfere with the functioning of the LLO secretion apparatus. Elucidation of the reason of the reduction of LLO hemolytic activity, for example by inhibiting initial binding to the erythrocyte membrane or subsequent step of pore formation, demands further experiments.

In the control experiment the complexation capability of OA or UA with listeriolysin was checked in nondenaturing polyacrylamide gel shift assay (Mori *et al*., 2004) using 2.5 µg of LLO and 0.08 µg or 0.12 µg OA or UA. No protein band shift was observed.

The ability of *L. monocytogenes* to form biofilms in the presence of OA/UA was estimated by crystal violet staining. OA and UA were added at 0.125, 0.25 or 0.5 minimal biofilm inhibitory concentration (MBIC). MBICs of both compounds were substantially higher than respective MICs (24 µg mL\(^{-1}\)). Both compounds inhibited cell adhesion to the surface of polystyrene multiwell plates. Decreases in biofilm formation of about 15% and > 60% were observed in the presence of 0.25 × MBIC and 0.5 × MBIC of OA/UA, respectively. In the presence of 0.125 × MBIC of OA or UA, the biofilm biomass was decreased by only about 12.6% and 8.8%,
respectively (Table I). The cell viability was reduced by 14% and 22% in the presence of 0.125 × MBIC of OA or UA and by 42% and 56% in the presence of 0.5 × MBIC of OA or UA, respectively (Fig. 3). Bacterial biofilms development is a multi-step process and their formation is influenced by a number of environmental factors including temperature, growth medium, pH and the nature of the surface (Moltz, 2005). Moreover, numerous molecular determinants are involved in the early stages of biofilm formation and the late stage of biofilm development among them is flagellum-mediated motility (O’Neil and Marquis, 2006). We previously observed that swarming-type, but not swimming-type, motility of *Pseudomonas aeruginosa* was severely inhibited by OA and UA (data not shown), therefore it may be speculated that OA/UA can diminish *L. monocytogenes* biofilm formation by negatively influencing its swarming movement ability.

It has been also established that peptidoglycan plays an important role in *L. monocytogenes* biofilm formation. Previous results from our laboratory showed that peptidoglycan metabolism was affected by OA/UA (Kurek et al., 2010). In the present study we found that tritium-labeled OA bound to isolated peptidoglycan and that the teichoic acids, long anionic polymers threading through peptidoglycan layers that are crucial in protecting bacteria against harmful molecules and environmental stresses (Xia et al., 2010) only partially inhibited this binding (Table II). Taken together these results suggest that changes induced by OA and UA in the peptidoglycan structure of *L. monocytogenes* influence biofilm formation.

In conclusion, we have demonstrated that oleanolic and ursolic acids affect the virulence factors of *L. monocytogenes*. Both triterpene acids inhibit listeriolysin O activity without influencing its secretion and they also diminish the ability of this species to grow in biofilms.

**Acknowledgements**

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**Table I**

<table>
<thead>
<tr>
<th>OA/UA concentration [MBIC]</th>
<th>A&lt;sub&gt;570&lt;/sub&gt;</th>
<th>OA</th>
<th>UA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.493 ± 0.049</td>
<td>2.493 ± 0.049</td>
<td></td>
</tr>
<tr>
<td>0.125 × MBIC</td>
<td>2.179 ± 0.038*</td>
<td>2.293 ± 0.015</td>
<td></td>
</tr>
<tr>
<td>0.25 × MBIC</td>
<td>2.097 ± 0.042*</td>
<td>2.175 ± 0.021*</td>
<td></td>
</tr>
<tr>
<td>0.5 × MBIC</td>
<td>0.984 ± 0.037*</td>
<td>0.861 ± 0.032*</td>
<td></td>
</tr>
</tbody>
</table>

The results are the means of three independent experiments ± standard deviation. MBICs of both OA and UA were 24 µg mL⁻¹.

* Difference statistically significant (*p < 0.05 vs control).

**Table II**

<table>
<thead>
<tr>
<th>[µg mL⁻¹]</th>
<th>Radioactivity of peptidoglycan (dpm per sample)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>- Teichoic acids</td>
</tr>
<tr>
<td>0.5</td>
<td>322.52 ± 55.32</td>
</tr>
<tr>
<td>2.5</td>
<td>469.33 ± 57.20</td>
</tr>
<tr>
<td>5</td>
<td>573.99 ± 35.19</td>
</tr>
<tr>
<td>10</td>
<td>875.92 ± 31.12</td>
</tr>
</tbody>
</table>

The presented values are the means of three independent experiments after subtraction of the background value ± standard deviation.

* Difference statistically significant (*p < 0.05).

**Fig. 3.** Effect of OA and UA on the viability of *L. monocytogenes* cells in a biofilm. The results are the means of three independent experiments and the error bars indicate standard deviations (*p < 0.05 vs control).

**Literature**


Grudniak A.M., A. Kurek, J. Szarlak and K.I. Wolska. 2011 Oleanolic and ursolic acids affect the expression of the cysteine

Fig. 3. Effect of OA and UA on the viability of *L. monocytogenes* cells in a biofilm. The results are the means of three independent experiments and the error bars indicate standard deviations (*p < 0.05 vs control).


Iron Shortage and Bile Salts Play a Major Role in the Expression of \textit{ompK} Gene in Vibrio anguillarum

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Abstract

The outer membrane protein K, OmpK first identified in \textit{Vibrio parahaemolyticus} has been shown to be a receptor for a broad host range vibriophage KVP40 infecting members of the \textit{Vibrionaceae}. In the study, the effect of culture conditions on the expression of \textit{ompK} in \textit{V. anguillarum} was studied using real-time PCR. The expression increased significantly in the presence of bile salts and iron chelating agent 2, 2’ bipyridine, suggesting a role for this protein in bile resistance and also in iron acquisition by \textit{V. anguillarum}. OmpK induction by iron limitation and the presence of bile salts was reconfirmed by western blot technique after growing the cells in trypticase soy broth supplemented with bile salts, blood and 2, 2’ bipyridine. We surmise that the expression of OmpK protein of \textit{V. anguillarum} is bile salt and iron chelating agent-dependent.

Keywords: \textit{Vibrio anguillarum}, OmpK expression, Real-time PCR, Western blot, bile salt, iron deprivation

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Introduction

\textit{Vibrio anguillarum}, a marine bacterium belonging to the family \textit{Vibrionaceae} is a major bacterial pathogen of marine and estuarine fish throughout the world (Myhr et al., 1991). \textit{V. anguillarum} infection results in fatal haemorrhagic septicaemia in marine fish when the immune system of fish is compromised or when the mucosal surfaces are damaged (Croxatto et al., 2007).

The outer membrane of Gram-negative bacteria consists of phospholipids and proteins. The proteins make up to 50% of the outer membrane mass. In recent years, these outer membrane proteins have attracted attention, since they have an important role in the host-bacteria interaction in adherence, uptake of nutrients including iron from the host, and subverting host defense mechanisms (Li et al., 2008).

To date, studies on \textit{V. anguillarum} outer membrane proteins have focused on OmpU, the major outer membrane protein present in large quantities and OM2, the induced outer membrane protein, which is known to be linked with virulence of the organism (Pedersen et al., 1997; Simón et al., 1996; Wang et al., 2003). However, more interest has been given to the role of membrane proteins involved in iron uptake such as FetA and FvtA. Recently it was demonstrated that the expression of these proteins decreases spectacularly with iron readiness (Naka and Crosa, 2012). Some Omps play a role in efflux of salinity, therefore their expression increases with the rise of salt concentration (Davey et al., 1998, Kao et al., 2009).

Although the presence of OmpK has been demonstrated in \textit{V. cholerae}, \textit{V. parahaemolyticus}, \textit{V. harveyi} and \textit{V. alginolyticus} (Inoue et al., 1995a), only the protective efficacy of \textit{V. alginolyticus} OmpK against fish vibriosis caused by \textit{V. alginolyticus} has been reported (Qian et al., 2008). Therefore in this study, we attempted to look for OmpK at DNA and protein level; in addition, we also studied the expression of \textit{ompK} gene by real-time PCR and the effect of different culture conditions on the expression to get an insight on the role of this outer membrane protein in \textit{V. anguillarum}.

Experimental

Materials and Methods

Bacterial strain and growth condition. \textit{V. anguillarum} NB10 kindly provided by Dr. Sahul Hameed (Abdul Hakeem College, India) was maintained at...
–80°C in nutrient broth containing 30% glycerol (Sanyo Corporation, Japan). The culture was set up by inoculating 5 ml of sterile trypticase soya broth (TSB) supplemented with 1% NaCl and incubated overnight at 28°C.

**Nucleic acid extractions.** DNA extraction was carried out from 1 ml of *V. anguillarum* culture using the method described by Ausubel et al., 1995. The DNA pellet was resuspended in Tris-EDTA buffer (10 mM Tris-Cl, 1 mM EDTA, pH 8). RNA extraction and purification was done by RNeasy Protect Bacteria Mini Kit (Qiagen, USA) according to the protocol described by the manufacturer. Extracts were subsequently treated with DNase I (Fermentas International Inc., Canada) according to manufacturer’s guidelines to remove any remaining DNA.

**Primer design.** To study the *ompK* gene of *V. anguillarum*, the primers F-k502: GGTCTCCAAAATTTCAACCAG and R-k699: TTTAAGGCGTAGCCAACACAG were designed using the Primer3 software (http://biotools.umassmed.edu/bioapps/primer3 www.cgi). The primers were used to amplify the *ompK* gene (GenBank accession no. FJ705222.1). The primers F-rpoA: CGTAGCTGAAAGCAGATGA and R-rpoA: AGCTGGAACATAACACGA were previously reported by Defoirdt et al., 2007 were used to amplify rpoA, which served as an internal standard gene.

**Effect of growth conditions.** The experiment was carried out in 100 ml TSB inoculated with 1 ml overnight culture followed by incubation at 28°C. To study the effect of NaCl on *V. anguillarum* growth, bacteria were grown in TSB supplemented with the following NaCl concentrations: 1% (control), 2%, 3% and 4%. To study the effect of bile salts, the culture was grown in media containing 0.1%, 0.2%, and 0.4% bile salts, and also grown in media free of bile salt (control). The effect of blood was determined by using 1%, and 2% of fresh blood; culture grown in media without blood were taken as control. The effect of the iron chelating agent 2, 2’-bipyridine was studied by growing the bacteria with or without 20 mM 2, 2’-bipyridine. The effect of a combination of 1% blood and 100 mM of 2, 2’-bipyridine was also studied.

**cDNA synthesis.** Reverse transcription was carried out according to the protocol of Fermentas Life Sciences (Fermentas International Inc., Canada). Briefly, the RNA was reverse transcribed to cDNA from 2 μg of RNA using 2 μl of reverse primer (100 ng/μl) and 0.5 μl of RevertAid H minus (Fermentas International Inc., Canada). Reverse transcription was carried out according to the protocol of Fermentas Life Sciences in the production of *ompK* gene were harvested by centrifugation at 4000g for 10 min and were lysed by discontinuous sonication on ice at 20–30 W with six 10 s bursts followed by centrifugation at 5000g for 20 min to remove the unlysed cells. Cell lysates were resolved in 15% SDS-PAGE (without staining) and electrotransferred onto nitrocellulose blotting membrane (Pall, New York, USA) at a constant voltage of 50 V for 1 h according to our previous article (Hamod et al., 2012). The membrane was blocked with 3% bovine serum albumin (BSA) overnight at 4°C. This was followed by three times washes with phosphate buffer saline containing 0.05% Tween-20 (PBST) followed by 3 washes with phosphate buffer saline (PBS) (1 min each time). The protein bands on the membrane were allowed to react with the rabbit anti-OmpK serum (Sigma, Missouri, USA) diluted 1:1000 for 1 h according to our previous article (Hamod et al., 2012) diluted 1:100 with gentle agitation at room temperature for 1 h, followed by washing and further incubation with horse radish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin G antibodies (Sigma, Missouri, USA) diluted 1:1000 for

**Real-time PCR analysis.** Quantification of expression level of the *ompK* gene was done by real-time PCR using 7300 Real-time PCR System (Applied Biosystems, Foster city, USA). Validation of the real-time PCR was done by amplifying serial dilutions of cDNA synthesized from 1 μg of RNA isolated from bacterial samples (Livak and Schmittgen, 2001). to carry out relative quantification using the 2^-ΔΔct_ formula.

The appropriate primer concentrations (200 nM for *ompK* and 300 nM for rpoA) were used for thermal cycling which included an initial activation at 50°C for 2 min, initial denaturation at 95°C for 10 min followed by 45 cycles of denaturation at 95°C for 15 s, primer annealing at 60°C for 45 s and elongation at 72°C for 30 s and a melting-curve program (95°C for 15 s and 60°C for 30 s). This was performed for each gene to check for amplification of the targeted fragments. Each reaction was performed in triplicate and the average value was considered for data analysis and statistical validation. Amplification data was recorded at the end of each elongation step.

**Analysis of relative gene expression was performed using the 2^-ΔΔct_ method of Livak and Schmittgen (2001).** The expression of the target genes was normalized to the endogenous control by calculating ΔCt, and expressed relative to a calibrator by calculating ΔΔCt (ΔΔCt = ΔCt_target – ΔCt_rpoA). The control values (5 h time interval, 0.5% NaCl, 0% bile salt, iron and 2, 2’-bipyridine) of each experimental condition were used as calibrator. The expression level of the *ompK* gene in each condition of the study was tested by an independent sample student t-test for calculating ΔCt data. Significance level was taken at 5%.

**Western blotting.** Bacteria grown under different culture conditions that resulted in a significant difference in the production of *ompK* gene were harvested by centrifugation at 4000g for 10 min and were lysed by discontinuous sonication on ice at 20–30 W with six 10 s bursts followed by centrifugation at 5000g for 20 min to remove the unlysed cells. Cell lysates were resolved in 15% SDS-PAGE (without staining) and electrotransferred onto nitrocellulose blotting membrane (Pall, New York, USA) at a constant voltage of 50 V for 1 h according to our previous article (Hamod et al., 2012). The membrane was blocked with 3% bovine serum albumin (BSA) overnight at 4°C. This was followed by three times washes with phosphate buffer saline containing 0.05% Tween-20 (PBST) followed by 3 washes with phosphate buffer saline (PBS) (1 min each time). The protein bands on the membrane were allowed to react with the rabbit anti-OmpK serum developed by us as described in our previous work (Hamod et al., 2012) diluted 1:100 with gentle agitation at room temperature for 1 h, followed by washing and further incubation with horse radish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin G antibodies (Sigma, Missouri, USA) diluted 1:1000 for
Expression of ompK in V. anguillarum

1 h. The reaction was carried out by dipping the membrane in tetramethylbenzidine solution (Merck, India) after thorough washing with PBS, stopped by rinsing with distilled water and visualized.

**Results**

**Effect of culture conditions on the expression of ompK gene.** Study on the effect of varying salt concentrations on growth and OmpK expression of *V. anguillarum* NB10 showed that NaCl concentrations up to 3% had a minimal effect. Expression of *ompK* at 2% NaCl (2.74 fold) was observed to be higher than at 1%, 3% and 4% of NaCl. The growth of *V. anguillarum* NB10 seemed to decrease with increasing bile salt concentration in the medium while the expression of *ompK* increased with increase in bile salt concentration (Fig. 1a). Expression of *ompK* gene was the highest at 0.4% bile salt concentration (5.8 fold) (Fig. 1a). Supplementation of iron chelating agent 2, 2'-bipyridine increased *ompK* 5.7 fold (Fig. 1b). On supplementation of culture medium with fresh human blood, the expression of *ompK* decreased (Fig. 1b). When both 20 mM 2, 2'-bipyridine and blood were supplemented, *ompK* expression was reduced to 2.3 fold compared to 5.7 fold in the presence of 2, 2'-bipyridine alone (Fig. 1b).

**Western blot analysis.** Positive bands with molecular weight of 28 KDa were seen only in the lanes containing cell lysates of *V. anguillarum* grown with 0.4% of bile salts, 20 mM bipyridine, and 1% of blood with 100 mM bipyridine (Fig. 2). No bands appeared in the other lanes containing the cell lysates of *V. anguillarum* grown with 4% NaCl, 2% blood supplement or bacteria grown in TSB without any supplementation.

**Discussion**

*V. anguillarum* is a causative agent of vibriosis in finfish and invertebrates such as bivalves and crustaceans. Vibriosis results in massive losses to the aquaculture
The protein in the cell lysate of V. harveyi was induced with increased synthesis occurring only under certain circumstances (Koebnik et al., 1995a, 1995b). Two iron uptake mechanisms have been reported in this organism viz. Iron uptake mechanisms are involved in conferring virulence to V. anguillarum. Two iron uptake mechanisms have been reported in this organism viz. production of anguibactin and vanchrobactin (Lemos et al., 1988). We observed that expression of ompK was induced in the presence of iron chelating agent 2’,2’-bipyridine and this is partially offset if blood is present in the medium (Fig. 1b), which probably suggests that OmpK in V. anguillarum responds to changes in iron level in the environment. Therefore, V. anguillarum strain was grown in TSB medium supplemented with 20 mM of 2’,2’-bipyridine, 0.4% bile salt, 4% NaCl, 2% of blood and combination of bipyridine and blood for the detection of OmpK protein by Western blot. A band with 28 kDa (Fig. 2) was detected only in the case of purified OmpK protein. The protein in the cell lysate of V. anguillarum was undetected by Western blot. It has been reported that expression of OMP is related to environmental conditions like temperature, salinity, availability of nutrients and oxygen (Nandi et al., 2005).

Real-time PCR technique, a reliable quantitative method for measuring gene expression (Nielsen and Boye, 2005) was used in this study to determine the effect of varying environmental factors such as salt concentration, bile salt concentration and iron chelating agent on the expression of ompK gene in V. anguillarum. V. anguillarum, a marine bacterium, well recognized as a pathogen of marine and estuarine fish, encounters different osmotic environments. The outer membrane proteins have a crucial role in maintaining the osmotic state of the organism during any salt stress. In this study growing V. anguillarum at salt concentrations higher than 2%, which is the optimum for its growth, did not upregulate ompK expression suggesting that ompK probably is not involved in osmoregulation. On the contrary, using the proteomic approach of Kao et al., 2009 showed that both ompU and ompW are highly expressed at 3.5% NaCl and are possibly involved in osmoregulatory function in V. anguillarum.

V. anguillarum being a fish pathogen, when ingested by fish is likely to be exposed to bile in the fish gut and therefore might be in possession of mechanisms for survival in the gut environment. Our study shows that expression of ompK is upregulated in the presence of bile salts. Though growth seemed to be suppressed at 0.4% bile salt (data not shown), expression of ompK was 5 fold higher (Fig. 1a) suggesting a role for this protein in bile resistance. Wang et al., 2003 showed OmpU to play a role in bile resistance in V. anguillarum. In Escherichia coli two outer membrane proteins OmpF and OmpC confer bile salt resistance. While OmpF is sensitive to bile salt concentration changes at low temperature and salinity, OmpC responds to alteration in bile salt concentration at elevated temperature (Nikaido and Rosenberg, 1983). Similarly, we suggest that in V. anguillarum, in addition to OmpU (Wang et al., 2003), OmpK may also be playing an important role in bile salt resistance and its uptake.

Polyclonal antibody was raised against the purified recombinant OmpK protein in rabbit in our work (Hamod et al., 2012). Surprisingly a positive band was detected only in the case of purified OmpK protein. The protein in the cell lysate of V. anguillarum was undetected by Western blot. It has been reported that expression of OMP is related to environmental conditions like temperature, salinity, availability of nutrients and oxygen (Nandi et al., 2005).
Expression of ompK in V. anguillarum

visualized in the case of V. anguillarum strain grown in bile salt, bipyridine and combination of blood and bipyridine. This result further confirms the role of OmpK protein in bile salt resistant and iron uptake.

Acknowledgment
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Literatures


Original Paper

Introduction

Synbiotic is a combination of probiotic and prebiotic, which can synergistically promote the growth of beneficial bacteria or newly added species in the colon (Macfarlane et al., 2008). During the development of new synbiotic products it is very important to ascertain prebiotic and probiotic interactions and influence of prebiotic on probiotic growth and antibacterial activity. Prebiotics are described as non-digestible poly- or oligosaccharides that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of beneficial bacteria in the colon (Gibson and Roberfroid, 1995). Prebiotics themselves have many health promoting properties. Moreover, there is evidence that they can help modulate the growth of gut microbiota and stimulate bacteriocin production (Kunova et al., 2011; Patel and Goyal, 2012).

Inulin, palatinose and α-cyclodextrin used in this work are generally recognized as safe (GRAS) food additives (Wagner et al., 2006; Holub et al., 2010). Inulin is a linear polymer consisting of β-(2→1)-fructosyl-fructose linkages (Roberfroid, 2007). Palatinose is a reducing sugar, composed of glucose and fructose moieties, joined with α-1,6-glycosyl bonds. Commercially palatinose is made from sucrose by enzymatically reorganizing glycosyl bonds (Holub et al., 2010). Cyclodextrins (CDs) are circular oligosaccharides, composed of glucose residues linked by α-1,4-glycosyl bonds. The hydrophilic part of the molecule is faced to the outside of the ring, and the hydrophobic part is inside the ring (del Valle, 2004; Wagner et al., 2008). Commercially cyclodextrins are produced from a starch. These molecules are widely used in pharmacy, chemistry and food industry due to their ability to form inclusion complexes (del Valle, 2004). Although βCD is more industrially used due to its lower price, αCD is characterized by better solubility in water (140 mg/ml) than βCD (18 mg/ml, 25°C) (Szejtli, 2004).

Inulin, palatinose and α-cyclodextrin can act in many health promoting ways, like stimulating immune system, reducing the amounts of pathogenic (Bacillus sp., Escherichia coli, Campylobacter jejuni, Salmonella et al., 2006; Patel and Goyal, 2012).
Porphyromonas gingivalis, Lactobacillus delbrueckii, Lactobacillus curvatus have been made. However, not many investigations of these properties could be useful parts of synbiotics for diabetic patients. This species should be able to ferment inulin, palatinose, and increase the amounts of bifidobacteria (Jo et al., 2007). The analysis of L. casei strains, was used as control (Saminathan et al., 2010). The sources of Lactic Acid Bacteria (LAB) were probiotic yogurts: “Actimel” and “Bifi”. For the isolation of strains, 1 g of each yogurt were added to 99 ml of 0.85 % sterile saline solution. Tenfold serial dilutions of the samples were made and appropriate dilutions were streaked on de Man, Ragosa and Sharpe (MRS) agar plates (Kormin et al., 2001). The plates were incubated for two days under anaerobic conditions at 37°C by placing a gas pack in the anaerobic jar (Merck).

**Bacterial strains and growth conditions.** Six Lactobacillus sp. and two Lactococcus sp. type strains were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ): Lactobacillus acidophilus DSM 20079 (LA), Lactobacillus delbrueckii subsp. bulgaricus DSM 20081 (LD), Lactobacillus curvatus DSM 20010 (LC), Lactobacillus sakei subsp. sakei DSM 20017 (LS), Lactococcus lactis subsp. lactis DSM 20481 (LL) and L. lactis subsp. lactis DSM 20729 (LL2) (abbreviations of strains used further in the work are given in the brackets). Isolated and type strains of Lactobacillus sp. were cultured in MRS broth (Merck) or basal MRS (Saminathan et al., 2010). Type strains of Lactococcus sp. were grown in broth No. 92 (DSMZ culture medium list), which consisted of Tryptic Soy Broth (TSB, Merck), supplemented with 0.3% Yeast Extract (YE, Difco). Isolated strains were cultured aerobically or anaerobically, with (100 rpm) or without agitation, at 30°C. Type strains of L. acidophilus and L. delbrueckii subsp. bulgaricus were grown under anaerobic conditions, at 37°C. Other type strains were cultured aerobically, at 30°C.

**Oligosaccharide substrates.** Three different commercially available oligosaccharides were used in this study: inulin (Inl) (Alfa Aesar), palatinose hydrate (Pal) (TCI) and α-cyclodextrin (Ctd, αCD) (Merck). Stock solutions of these oligosaccharides were prepared in sterile distilled water and filter sterilized with 0.22 μm filters (Roth). The sterile oligosaccharide solutions were added to 99 ml of 0.85 % sterile saline solution. Tenfold serial dilutions of the samples were made and appropriate dilutions were streaked on de Man, Ragosa and Sharpe (MRS) agar plates (Kormin et al., 2001). The plates were incubated for two days under anaerobic conditions at 37°C by placing a gas pack in the anaerobic jar (Merck).

**Materials and Methods**

**Experimental**

**Strain isolation from yogurts.** The sources of Lactic Acid Bacteria (LAB) were probiotic yogurts: “Actimel” and “Bifi”. For the isolation of strains, 1 g of each yogurt were added to 99 ml of 0.85 % sterile saline solution. Tenfold serial dilutions of the samples were made and appropriate dilutions were streaked on de Man, Ragosa and Sharpe (MRS) agar plates (Kormin et al., 2001). The plates were incubated for two days under anaerobic conditions at 37°C by placing a gas pack in the anaerobic jar (Merck).

**Genomic DNA extraction.** Genomic DNA of isolated strains was extracted using Genejet DNA Extraction Kit (Thermo Fisher Scientific). Universal bacterial 16S rDNA primers (27F and 1492R, Thermo Fisher Scientific) were used for PCR reactions. Reaction products were sequenced at the Sequencing Center of the Institute of Biotechnology. In silico data analysis was performed using the NCBI Blast programme (http://blast.ncbi.nlm.nih.gov/Blast.cgi).
In order to determine the variety of strains isolated from yogurts, we performed PCR reaction with all strains genomic DNA and universal BOX primers. Results were analyzed by agarose gel electrophoresis (Sambrook and Russell, 2001).

**Antibacterial activity assay.** The antibacterial activity of strains was determined using the spot-on-lawn method (Kormin et al., 2001) with modifications. The tested strain was grown overnight in the middle of Petri dish with an appropriate agar medium. Then the layer of agar medium with inoculated other presumably sensitive strain was poured on top of the grown culture. The agar media with inoculated strain was prepared by adding 20% of 1.2 OD culture inoculum into an appropriate agar media. Isolated and type strains were tested against each other.

**Agar well diffusion assay.** 0.5 cm diameter wells were made, using sterile plastic pipette tips, in the agar media with inoculated sensitive strain, prepared as described above. Culture samples were taken every two hours from the beginning of culture cultivation in liquid media. Samples were centrifuged and the serial twofold dilutions of the cell free supernatants were made. The amount of 100 µl of every dilution was poured into the prepared wells and plates were incubated overnight at the temperature appropriate for the used indicator strain. Clear zones round the wells after incubation indicate the inhibitory activity, which was expressed qualitatively as arbitrary units per milliliter (AU/ml). The arbitrary unit (AU) was defined as the reciprocal of the highest dilution able to produce a clear zone of growth inhibition of the indicator strain (Vera Pingitore et al., 2007).

**Disintegration of cells.** Samples (5 ml) of the grown cultures were taken every 6 h from the beginning of growth. Samples were centrifuged, cells were suspended in 5 ml of phosphate buffer (pH 6.0) and then disrupted with the ultrasound disintegrator. Obtained cell disintegration was filtered through 0.22 µm filter (Roth) or centrifuged (5 min, 14,000 × g). In order to determine the antibacterial activity, 100 µl of the filtered, centrifuged and untreated disintegrates were tested using agar well-diffusion assay using A20 strain as indicator.

**Identification of bacteriocins.** Primers (20 pairs in total) used in this work were described in scientific publications or constructed using typical well known bacteriocin encoding gene sequences from the Genbank database (http://www.ncbi.nlm.nih.gov.Genbank/index.html), are listed in Table I.

**Results**

**Isolation of bacteria from yogurts.** Since the morphology of bacteria grown on the MRS medium did not have any differences, 9 strains of Lactobacillus sp. isolates were randomly selected: 4 strains from “Actimel” yogurt (“Danone”) (A isolates) and 4 from “Bifi” yogurt (UAB “Rokiškio pienas”) (B isolates). The amount of Lactobacillus sp. bacteria in examined yogurts was estimated at 10^8 cells in 1 gram of yogurt. These data coincide with the information given by the producers.

For a more detailed identification of the isolates 16S rDNA analysis was made. Results have shown that the isolated strains are most similar to L. casei and L. paracasei subsp. tolerans species (reliability of BLAST analysis results is 100%). Implementation of BOX-PCR reaction, used to estimate the variety of bacterial strains among isolates, has revealed distribution of strains in the profiles, fully complying with strains origin from different yogurts (data not shown).

**Antagonistic activity assay of isolated strains.** 3 strains from group A and 3 strains from group B were selected for the antagonistic activity assay. Using spot-on-lawn method it was determined that all isolated lactobacilli strains were able to produce clear zones inhibiting the growth of the strains, used as indicator (isolated strains were tested against each other). Results, given in Table II, reflect the antagonistic spectrum of the tested strains. The obtained results allow us to assert that strains belonging to groups A and B produce antibacterially active compounds. The size of the formed clear zones (Table II.) shows that the antibacterial compound of B group strains is more active or a larger amount of it is produced.

**Antibacterial activity of isolated and type strains.** Antibacterial activity of 3 isolated (A11, B13, A20) and 6 type strains against each other was determined and phenotypically evaluated (Table III.). Results have shown that LL2 strain possesses the highest antibacterial activity among type strains. The antibacterial spectrum and ability to inhibit the growth of tested strains of this strain did not differ from antibacterial spectrum and activity of isolated lactobacilli strains A11, A20 and B13. However, only LD strain had no antibacterial activity against any of the tested strains and was sensitive to all antibacterial compounds secreted by the tested strains.

**Identification of bacteriocins produced by isolated and type strains.** One of the possibilities to identify secreted bacteriocins is a search of bacteriocin encoding genes in the genomes of analyzed strains. For this purpose we used 20 pairs of primers constructed in accordance with genes of well known bacteriocins (GeneBank Database) or selected from scientific publications (Table I.). All primers were used for PCR reactions with genomic DNA of 15 strains (9 isolated and 6 type strains). The results of the size and sequence analysis of the obtained PCR products can be divided into five groups: 1) Two PCR products compatible with genes encoding nisin by their size and results of
BLAST analysis (primers 3 and W, genomic DNAs of LL2 and LL strains respectively, Table I). 2) PCR products, obtained with 5 primers constructed for detection of new bacteriocins of *L. casei* ATCC334 (63, 86, 93, 405, 406) (Kuo et al., 2013) and genomic DNAs of isolated strains. Sequences of the products had homology with the sequences of new bacteriocins, mentioned before. 3) PCR products obtained with primers constructed according to prebacteriocin genes found in the genome of *L. casei* BDII strain (Genbank Database, GI: 385821700) (primers 92, 94 and 111 (Table I.), genomic DNAs of isolated strains), which were compatible with

<table>
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<tr>
<th>No</th>
<th>Primer (abbreviation)</th>
<th>Sequence</th>
<th>Tm, °C</th>
<th>Expected product (size, bp)</th>
<th>Source</th>
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<td>(Digaitiene et al., 2012)</td>
</tr>
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<td>75</td>
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<td>Nizin (590)</td>
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<td>Nizin</td>
<td>(Swetwiwathana et al., 2009)</td>
</tr>
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<td>Class II bacteriocin (≤ 300)</td>
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<td>13BACTP (13)</td>
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<td>69</td>
<td>Class II bacteriocin (≤ 300)</td>
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<td>14BACTP (14)</td>
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<td>(Kuo Y.-C. et al., 2012)</td>
</tr>
<tr>
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<tr>
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<td>16BACTP (16)</td>
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<td>69</td>
<td>Class II bacteriocin (≤ 300)</td>
<td>(Kuo Y.-C. et al., 2012)</td>
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the prebacteriocin genes by their size and sequence. However, more detailed in silico analysis of these products, using Bactibase database (http://bactibase.pfba-lab-tun.org/physicochem), has shown that, most likely, these genes do not encode bacteriocins. 4) PCR products, obtained with primers for well known bacteriocin genes and genomic DNAs of isolated and some of the type strains (primer for nisin (3) with genomic DNA of isolated, LL and LA strains; primer for lacticin 3147 (1) with genomic DNA of isolated and LD strains; primer for curvacin A (CA2) with genomic DNA of isolated and LD strains), which were not compatible with these bacteriocin genes by their size and sequence analysis, but were identified as hypothetical or unrelated function proteins. 5) No PCR products were obtained with 7 primers for the well known bacteriocin genes (pediocin PA (2), sakacin P (4 and SP), sakacin A (SA), lactacin S (L), acidiocin 8120 (AT), curvacin A (CA) (Table I)) and genomic DNAs of all strains.

**Ability of the isolated and type strains to assimilate OS.** Considering the results of BLAST and BOX-PCR analysis, only strains A11 and B13 were used for further experiments, as they represent different groups of BOX-PCR profiles and possess the highest antibacterial activity among the strains of their groups (Table II.).

Data from the graphs presented in Fig. 1. (A and B) suggest that in all cases both A11 and B13 strains most weakly assimilate palatinose hydrate. On the other hand, inulin is poorly assimilated only by strain A11 (Fig. 1A). The growth of strain B13 with inulin is equivalent to the growth of this strain with glucose, except that the exponential growth phase begins about 4 h later (Fig. 1B). The increase of OS concentration does not influence the growth of strains.

Analyzing the influence of different carbon sources (glucose, inulin and palatinose) on the type strains of Lactobacillus sp. and Lactococcus sp., the following was determined:

1. In the medium with glucose as an only carbon source L. lactis subsp. lactis strains (LL and LL2) grew faster than strains LS and LA. LL and LL2 strains reached the stationary phase after 6 and 8h respectively (Fig. 1C and D), while strains LS and LA reached this phase of growth after 14 and 28 h respectively (Fig. 1E and F). Evaluation of growth time and cell density at the beginning of the stationary phase suggests that strain LL2 grows fastest in the medium with glucose.

2. Strain LL assimilates palatinose best among the type strains (Fig. 1C). The culture reaches cell density of 1.2 OD in the beginning of stationary phase after 14 h of growth, while assimilation of inulin is ineffective – maximum reached OD was 0.5.

3. The assimilation of inulin and palatinose by strain LS is completely ineffective (Fig. 1E).

4. Strain LL2 equally poorly assimilates both inulin and palatinose. Only 0.5 OD cell density is reached in the beginning of the stationary phase, after 5 h of growth (Fig. 1D).

5. LA also ineffectively assimilates inulin and palatinose. The stationary phase with the cell density of 0.5 OD is reached after 12 h in the medium with inulin and only after 24 h when growing with palatinose (0.45 OD) (Fig. 1F).

The MRSb medium becomes opaque after addition of αCD and it becomes impossible to track the alterations of OD. Therefore, the influence αCD on the growth of strains was analysed only with strains LL and LL2. Results (Fig. 1C and D) have shown that αCD is actively assimilated by LL2 strain.

**Evaluation of antibacterial activity of isolated strains.** Antibacterial activity of culture supernatants was evaluated by the well-diffusion method during the growth (Vera Pingitore et al., 2007). Strain A20 was used as indicator for the evaluation antibacterial activity of the isolated strains, while testing on agar medium by spot-on-lawn method it demonstrated the highest.

---

**Table II**

Results of antibacterial spectrum of isolated strains.

<table>
<thead>
<tr>
<th>Sensitive strain</th>
<th>A11</th>
<th>A15</th>
<th>A20</th>
<th>B1</th>
<th>B4</th>
<th>B13</th>
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<tr>
<td>A11</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>+++</td>
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</tbody>
</table>

“+” – diameter of the clear zone around the secreting culture exceeds 1.5 mm; “++” – clear zone ≤ 2.5 mm, “+++” – zone > 2.5 mm.

---

**Table III**

Results of antibacterial activity of isolated and type strains.

<table>
<thead>
<tr>
<th>Sensitive strain</th>
<th>A11</th>
<th>B13</th>
<th>A20</th>
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<th>LL</th>
<th>LL2</th>
<th>LC</th>
<th>LS</th>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>B13</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
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<tr>
<td>A20</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>+</td>
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<tr>
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<td>+</td>
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</table>

“+/-” – clear zone is invisible only by the borders of the secreting culture; “+” – clear zone is hardly visible, ≤ 0.5 mm; “++” – clear zone ≤ 1.5 mm; “+++” – clear zone > 2.5 mm.
sensitivity to the antibacterial compounds secreted by other isolated strains (Table II). However, no antibacterial activity was detected while testing supernatants from the cultures grown in the liquid medium. The negative results could be caused by:

- interaction between antibacterial compound and cell surface;
- accumulation of antibacterial compound inside the cell;
- low concentration of the secreted antibacterial compound.

Accordingly, the cultivation of strains A11 and B13 was repeated after addition of 0.5% and 1% of Tween-80, which should reduce the interaction between proteins and cell surface and also enlarge the amount of antibacterial compound in the culture supernatant (the initial concentration of Tween-80 in the MRS medium is 0.1%). However, no clear zones were detected after repeated evaluation of antibacterial activity of culture supernatants.

Culture samples (5 ml) were taken every 6 hours from the beginning of growth. The samples were centri-
fuged and cells were disintegrated. Antibacterial activity of filtered, centrifuged and untreated cell disintegrates was tested by the well-diffusion assay, but no antibacterial activity was detected.

Aiming to increase the amount of antibacterial compound, A11 and B13 strains were grown in the liquid medium till the end of exponential growth phase. 50 ml of every culture supernatants were salted out with 80% saturation of ammonium sulphate. Suspension of proteins was dialyzed in the phosphate buffer and concentrated up to 2 ml of volume. The antibacterial activity of the crude protein extracts was tested by the well-diffusion method, but no definite clear zones were detected.

Another factor that could influence the production of bacteriocins is a deficiency of certain minerals. So strains A11 and B13 were grown in medium supplemented with combination of minerals, which were optimized in our laboratory to the growth and bacteriocin production of bacteria from other genera. It was shown that addition of NaHSO\textsubscript{4} and MnSO\textsubscript{4} to the medium (MnSO\textsubscript{4} is an ingredient of MRSb medium) almost completely inhibits the growth of strain A11, but in the case of strain B13, these minerals only prolong the lag phase of growth. Other used combinations of minerals practically have no influence on the growth of the strains. However, culture supernatants did not possess any antibacterial activity either.

It is known that pH of the growth medium strongly influences to the bacteriocin expression. But no antibacterial activity in culture supernatants after changing the pH of the growth medium from 5.6 to 7.2 was detected.

**OS influence on bacteriocin secretion.** Strain A20 was replaced with LL as indicator strain (Table III.) and the antibacterial activity of A11 and B13 culture supernatants repeatedly grown with different carbon sources (glucose or 1% OS) was evaluated by well-diffusion assay. In this case about 2 mm of diameter unclear transparency zones around the wells with the culture supernatants after 12 and 14 h of growth were detected. The activity of secreted putative bacteriocins was minor – after diluting the supernatants only two times the activity was not detected. LL2, LS, LD and LC strains were also used as indicators but no clear zones had been detected around the wells with A11 and B13 strains grown in liquid medium culture supernatants either.

No antibacterial activity was detected in culture supernatants while analyzing the influence of OS on the type strains, although all type strains, sensitive to the grown strains respectively (Table III.), were used as sensitive. Positive results were obtained only with strains LL2 and LA while using LS and LC strains as indicators.

The antibacterial activity of strain LL2 culture supernatants was tested using LC strain as indicator, as it is characterized by the highest sensitivity to the bacteriocin secreted by LL2 strain (Table III). After 6 h of growth of this strain in the medium with glucose or α-cyclodextrin, the antibacterial activity of culture supernatants was two times higher (80 AU/ml) when grown with αCD, than with glucose (40 AU/ml), although the cell density of the culture grown with glucose at that moment was almost two times bigger, than grown with αCD (1.5 OD and 0.8 OD respectively) (Fig. 1G). These results show that αCD positively influences bacteriocin production or activity. While growing the strain in the medium with inulin or palatinose, the antibacterial activity was detected only after 8 h (20 AU/ml). That suggests that strain LL2 is not capable of assimilating palatinose and inulin and, therefore, the antibacterial activity of culture supernatants is very weak (Fig. 1G). It should be noted that culture supernatant, obtained after 10 h of growth with αCD, maintains the same activity as after 8 h of growth (320 AU/ml), and while growing in the medium with glucose, the activity of culture supernatant decreases two times (Fig. 1G).

Strain LS is characterized by high sensitivity to the antibacterial compound produced by strain LA (Table III). Due to this fact, this strain was used as indicator for evaluation of the antibacterial activity of strain LA grown in the liquid medium. Besides, both these strains can be cultivated in the MRS medium. Diverse influence of OS on the antibacterial activity of LA strain, grown with different carbon sources – glucose, palatinose and inulin was shown (Fig. 1H). During the growth of this strain, the antibacterial activity of culture supernatant occurred in the 8th hour of growth. Culture supernatants of 16, 20 and 24 hours possessed the highest activity (320 AU/ml) during the exponential growth phase of the investigated strain. Cell density of the LA culture after 8 h of growth in the medium with glucose or with palatinose practically does not differ. Although the antibacterial activity of the culture supernatant with palatinose is two times lower, the activities becomes equal after 28 h of growth, when the culture ends the exponential growth in the medium with glucose and is already in the stationary phase in the medium with palatinose (Fig. 1F and H). The cell density of the culture is equal (0.5 OD) after 12 h of growth in the medium with glucose and in the medium with inulin, but the antibacterial activity of the culture supernatant with inulin is 8 times lower. The antibacterial activity decreases two times after 16 h of growth in the medium with inulin and becomes undetectible after 20 h.

It should be noted that the growth of the LA strain measuring the cell density of the cultures in the media with inulin and palatinose only slightly differs (1.25 times) (Fig. 1F). However, the antibacterial
activity of culture supernatants with palatinose at those hours, when it is possible to evaluate the antibacterial activity of the supernatants with inulin, is at least 8 times higher (Fig. 1H).

**Discussion**

Three oligosaccharides were used in this work: palatinose and inulin, characterized by low caloricity and prebiotic properties, and α-cyclodextrin – practically not investigated as a component of synbiotics. The possibility to use these prebiotics in the development of synbiotics for healthy and especially for diabetic people, may be an effective mean for enhancement of the immune system and prevention of colon infections by pathogenic microorganisms.

Probiotics are the second component of synbiotics. One of the ways by which probiotics may influence proliferation of pathogenic bacteria in the gastrointestinal tract are bacteriocins, secreted by them. In this work, it has been demonstrated that *Lactobacillus* sp. strains, isolated from “Activia” and “Bifí” yogurts, are bacteriocin producers. In solid medium clear transparent zones, indicating growth inhibition of the sensitive strain, independently of whether glucose or OS were used as an only carbon source, were obtained. However, no antibacterial activity was detected in the liquid media. According to the literature, there was shown, that in many cases LAB strains exhibits antibacterial activity on solid but not in liquid media. However, further investigations showed that most of these bacteriocins could be produced also in liquid media, but only under optimized conditions (Maldonado-Barragán et al., 2009). We did not succeed to obtain crude extracts of these bacteriocins, although all methodical procedures usual for bacteriocin extraction were carried out. However, the obtained PCR products suggests that they may be the newly characterized bacteriocins of *L. casei* ATCC 334 (Kuo et al., 2013). Furthermore, analysis results of the ability to ferment the used carbon sources and influence of microelements on the growth of A11 and B13 strains confirm the distribution of isolated lactobacilli strains into two physiological groups, compatible with BOX-PCR profiles and isolation sources. These results suggest that B group lactobacilli have specific genetic systems enabling them to ferment inulin. However, palatinose is not assimilated by strains belonging to both A and B groups.

In the case of two type *L. lactis* and two type *Lactobacillus* strains it was determined that in the growth media with different OS as an only carbon source *L. lactis* subsp. *lactis* strains demonstrated good fermentation of α-cyclodextrin, almost equal to that of glucose. However, *Lactococcus* sp. strains differ in the efficiency of palatinose assimilation – LL stands out of all tested strains for the ability to ferment palatinose. It shows that this strain has specific enzymes, required for the transport and hydrolysis of this disaccharide. LS strain is totally unable to assimilate inulin and palatinose. On the other hand, LA strain ferments these OS inefficiently. Analysis of the influence of glucose, palatinose, inulin and α-cyclodextrin on the bacteriocin expression of LL and LL2 strains shows that in the case of glucose and α-cyclodextrin bacteriocins appear in the medium in the late stationary phase, which suggests that these antibacterial substances, according to PCR results – possibly nisin, are released into the growth medium during the lysis of cells.

The analysis of isolated lactobacilli in the medium with inulin as an only carbon source show that this OS can be used as a prebiotic to create synbiotics with B group lactobacilli isolated from yogurts. According to the analysis of *L. casei* genomes, carried out by Broad-bent and coworkers (2012), members of this species are able to ferment inulin. This and the results of 16S rDNA analysis suggest that our isolated strain B13 belongs to the *L. casei* species. Palatinose and LA strain could be used as synbiotics with the effective antibacterial activity. The same property would be characteristic of a synbiotic based on α-cyclodextrin and strain LL2.

The presented results offer the possibility to create synbiotics on the basis of probiotics – *Lactobacillus* sp. and *Lactococcus* sp. and prebiotics – palatinose, inulin and α-cyclodextrin, which could regulate not only the growth of different lactobacilli and lactococci, but also the efficiency of their antibacterial activity.

**Acknowledgements**

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**Literature**


Introduction

Urinary tract infections (UTI) are probably the most common bacterial infections. Bacteria responsible for UTI often originate from the faecal and perineal flora. Under normal circumstances, these bacteria are cleared from the urinary system by effective protective mechanisms. If, however, they overcome these mechanisms, they can colonize the lower urinary tract. Subsequent progress is determined by the host susceptibility and bacterial virulent factors (Kaper et al., 2004; Wullt, 2002).

Several in vitro and in vivo models of infection have provided insights into the progression of uropathogenic Escherichia coli (UPEC)-induced UTI (Justice et al., 2004). The unique ability to colonize different sites and to cause a UTI is potentially due, at least in part, to the versatile genome of UPEC, which are able to remodel their genetic repertoire by acquiring and losing virulence attributes.

A number of virulence determinants facilitate the ability of UPEC to colonize the urinary tract and exert cytotoxic effects, including type 1 fimbriae, P fimbriae, Dr adhesins, hemolysin, cytotoxic necrotizing factor 1, flagella, capsule polysaccharide, lipopolysaccharide O antigen, and iron transport systems (Hagan and Mobley, 2007; Slavchev et al., 2009). The distribution of virulence properties can also vary depending on host characteristics and type of infection. It has been found that these virulent genes are the distinguishing factor between pathogenic and non-pathogenic strains of E. coli (Bisi-Johnson et al., 2011). According to the conventional notion pathogens can develop from commensals by acquisition of virulence-associated genes located for example on pathogenicity islands or plasmids, and the commensal-to-pathogen shift in E. coli is bi-directional (Klemm et al., 2007).

The question is how many virulent genes are sufficient for UPEC to produce pathogenic properties? To give insight for answering this question, we studied many UPEC strains freshly isolated from patients with urinary tract infection after complete cultural, biochemical and serological identification, explored them...
for virulence factor genes and studied the pathogenic effect of their cell-free culture supernatant in vitro on tissue culture of Vero cells (green monkey kidney cells).

**Experimental**

**Materials and Methods**

**Sample collection.** A total of 15 UPEC isolates obtained in counts of >10^5 cfu/ml and in pure growth, from routine urine cultures (William and Parasuraman, 2001) of urinary tract infected patients presenting to the urology department in Ismailia university hospital, Suez canal university, Egypt. Identification of isolates was done using standard microbiological techniques (Cheesbrough, 1993). Serotyping for each pathogenic strain was performed by standard techniques as described by Ørskov and Ørskov (1984) using a commercial kit of Denka Seiken Co. LTD.

**Virulence factor profiling with PCR.** DNA was extracted from UPEC isolates cultured on Luria broth, with spin column according to the instructions of the manufacturer Bioer co. China. Amplification was performed in a 25 µl reaction mixture containing 8 µl of DNA template, 0.1 µl of each of nine primers (0.1–0.2 µM) from Bio Basic Inc., Canada and 8 µl of ready to go master mix 1X (containing thermostable DNA polymerase, dNTPs, reaction buffer with (NH₄)₂SO₄, MgCl₂ and Triton X-100, stabilizers) from Jena Bioscience co., Germany. PCR tubes were filled to 25 µl volume with PCR-grade water. The reaction was carried out in a PCR thermal cycler (Techgen co., USA) according to Takahashi et al. with modification in the following schedule: preheating at 94°C for 10 min followed by 32 cycles of denaturation at 94°C for 1 min, annealing at 60°C or 55°C, and extension at 72°C for 2 min, with a final extension at 72°C for 7 min (Table I). The PCR products were electrophoresed on 2% agarose gels, then stained with ethidium bromide and examined under UV transillumination (Takahashi et al., 2006).

**Tissue culture preparation (Vero cells).** Vero cells were supplied as 90% confluent monolayer sheet in growth medium with 10% new born calf serum. The confluent monolayer sheet was trypsinized with trypsin-versin 0.25% to separate the individual cells. The cells were distributed in tissue culture microtiter plate in which each well contained 10^4 cell/ml and were cultivated at 37°C in a 5% CO₂ atmosphere until the monolayer was confluent.

**Exotoxin production.** The selected strains were seeded in nutrient and syncase broth and then incubated for 24 h at 37°C in rotary shaker. Cultures were later centrifuged at 1800 xg for 30 min. The supernatants were then filtered through 0.22 mm Millipore filters. Two parts of exotoxins from syncase broth for each isolates were treated with heat and formalin.

**Endotoxin production.** Endotoxin was obtained following the procedure of Clugston and Nielson, in which bacterial suspension was harvested in PBS, and the toxin obtained after ten cycles of freezing and thawing (Clugston and Nielson, 1974). One part of endotoxin for each isolate was formalin treated.

**Toxigenicity test in cell cultures.** Toxin preparations were added to the cell culture in maintenance medium consisting of MEM supplemented with 2% fetal bovine serum (Caprioli et al., 1983; Slavchev et al., 2009). The assays were incubated for 24 h at 37°C. The control consisted of cell monolayer containing maintenance media, PBS, nutrient broth, syncase broth or 0.05% formalin alone after staining, cells were examined with inverted microscope (Olympus 1X70-S8F2, Olympus Optical Co., LTD. Japan).

**Results**

**Serotypes.** The 14 UPEC isolates belonged to 5 serogroups, while one of the isolate had no detectable reaction when available somatic and capsular antigens reagent were tested. This strain was identified as untypable. Five of these isolates have antigenic formula O78:K80 which represented 33% of the isolates and four isolates have antigenic formula O114:K90 (27% of the isolates). However, two of the isolates were with antigenic formula O142:K86 (13% of the isolates). Another two of the isolates were O164 antigen (13% of the isolates) and one isolate with formula O157 somatic antigen (7% of the isolates) and neither of them with unidentified capsular antigen (Table II).

**Virulent factor gene profiles of UPEC isolates.** Nine virulent factor genes were explored for all fifteen UPEC isolates. Of the fifteen strains obtained only one (strain 11) carried all tested virulent gene factors. However, two strains showed the presence of 78% of tested genes, and other two UPEC strains showed 67% of the nine virulent genes. Four strains showed evidence for pathogenicity by detection of the most virulence gene factors, which represent 56% of the tested genes. Among the rest of the UPEC strains, four strains showed the presence of 44% of virulence gene factors, and two strains showed fewer virulence gene, i.e. 22% and 33%, respectively.

The distribution patterns of virulent factor genes of our isolated UPEC strains are presented in Table II. The high prevalence one is traT gene, with 100% frequency within tested strains, followed by fyuA which was 93%. The third most prevalent gene was ompT gene with incidence of 87%. Genes with lower incidence among
Table I
Virulence factors genes primer sets used for PCR.

<table>
<thead>
<tr>
<th>VF gene</th>
<th>Product size (bp)</th>
<th>Annealing Temp. °C</th>
<th>Reverse sequence</th>
<th>Forward sequence</th>
<th>Primer used</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ibeA</td>
<td>170</td>
<td>55</td>
<td>5'-TGGTGCTCCGGGACAACCATGC-3'</td>
<td>5'-AGGCAGGTGCGCGGTAC-3'</td>
<td>ibe f/r</td>
<td>Johnson &amp; Stell, 2000</td>
</tr>
<tr>
<td>pap</td>
<td>336</td>
<td>60</td>
<td>5'-AGAGAGGAGCGACCTTTTACGGACA-3'</td>
<td>5'-GCAACAGCAACGTGTGACATCAT-3'</td>
<td>pap 3/4</td>
<td>Yamamoto et al., 1995</td>
</tr>
<tr>
<td>sfa/foc</td>
<td>410</td>
<td>60</td>
<td>5'-CGGAGGAAGTAAATACACCTGGCA-3'</td>
<td>5'-CTCCGGAGAATGGTGACCATCTTAC-3'</td>
<td>sfa/foc 1/2</td>
<td>Yamamoto et al., 1995</td>
</tr>
<tr>
<td>cnf1</td>
<td>498</td>
<td>60</td>
<td>5'-CATTCAGAGCTCCTGCCTCATTATT-3'</td>
<td>5'-AAGATGAGGTTCCTATGCAAGGAG-3'</td>
<td>cnf 1/2</td>
<td>Yamamoto et al., 1995</td>
</tr>
<tr>
<td>hly</td>
<td>1,177</td>
<td>60</td>
<td>5'-ACCATATAAGCGGTCATTCCCGTCA-3'</td>
<td>5'-AAAAGAGTAAAGCGTGTGCTGTCAT-3'</td>
<td>hly 1/2</td>
<td>Yamamoto et al., 1995</td>
</tr>
<tr>
<td>FyuA</td>
<td>880</td>
<td>55</td>
<td>5'-GCAGTAGGCACGATGTTGTA-3'</td>
<td>5'-TGATTAACCCCGCGACGG-3'</td>
<td>FyuA f/r</td>
<td>Johnson &amp; Stell, 2000</td>
</tr>
<tr>
<td>pil</td>
<td>207</td>
<td>60</td>
<td>5'-ATAACACGCCGCGCTAAAGCC-3'</td>
<td>5'-CATTCGCCGCTGCAAACCGCC-3'</td>
<td>fimH f/r</td>
<td>Tseng et al., 2001</td>
</tr>
<tr>
<td>ompT</td>
<td>559</td>
<td>60</td>
<td>5'-CCGGGTCATAGTGGTTCAT-3'</td>
<td>5'-ATCTAGCGAAGGAGGCGC-3'</td>
<td>ompT f/r</td>
<td>Johnson et al., 2000</td>
</tr>
<tr>
<td>traT</td>
<td>290</td>
<td>60</td>
<td>5'-CACAGTTCAGCCATCCGTGAG-3'</td>
<td>5'-GGTGTTGCGATGAGCAGCAG-3'</td>
<td>traT f/r</td>
<td>Johnson &amp; Stell, 2000</td>
</tr>
</tbody>
</table>

Table II
Distribution of virulent genes within UPEC serotypes and gene’s incidence rate.

<table>
<thead>
<tr>
<th>Strain No.</th>
<th>UPEC serotypes</th>
<th>Virulence genes (VG)</th>
<th>VG content (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Pf</td>
<td>Pap</td>
</tr>
<tr>
<td>1</td>
<td>O78 K80</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>O142 K86</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>O114 K90</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>O142 K86</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>O114 K90</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>O78 K80</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>O164 k Untypable</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>O157 k Untypable</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>O78 K80</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>O114 K90</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>11</td>
<td>O78 K80</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>12</td>
<td>O164 k Untypable</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>13</td>
<td>O114 K90</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>14</td>
<td>Untypable</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>15</td>
<td>O78 K80</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Gene’s incidence (%) | 80 | 13 | 53 | 40 | 13 | 87 | 100 | 93 | 27 |
the detected virulent gene factors were 27% foribeA and 13% for both hly and pap.

**Cytotoxic effects of UPEC on Vero cells.** Cytotoxic effects were observed in 15 culture filtrates and endotoxin preparations of UPEC strains. After incubation there were changes from monolayer spindle-shaped cells characteristic of normal Vero cells to abnormal shapes and arrangements, in addition to complete lysis which represented the major cytopathological effect (Fig. 1 and 2). These effects are expressed as percentage and cytopathological scores for comparison.

Percent of lysed cells on nutrient broth compared to synacase broth showed that significant higher numbers of lysed cells were observed in synacase broth as untreated exotoxin. However, treated filtrates, for all strains, showed significantly less virulence compared to the same synacase broth growth medium of untreated filtrate. Formalin treated-filtrates showed less pathologic effect compared to untreated filtrates, obtained from cells grown in synacase broth, or to heat-treated filtrates. All UPEC strains showed 100% cell lysed after incubation for 24 hrs except for strains 2 and 9 which showed 80% lysis when grown in synacase broth. Generally, we can conclude that cytopathic effects were varied among the UPEC strains when grown in nutrient broth but less virulent effect when compared to those recorded in synacase broth or heat-treated filtrates.

When exotoxins obtained from Synacase broth were treated with heat, the complete lysis of cells decreased to approximately 50–70% lysis with debris. The remaining cells showed some distortions of the cytoplasm with conserved nuclei and clumped chromatin but still even a short rim of cytoplasm around. When treating the UPEC exotoxins obtained from synacase broth with formalin, the cell lysis decreased to about 10–20% in all UPEC strains and the monolayer kept its general architecture. Few cells show slight enlargement, and some showed loss of nuclear or cytoplasmic membrane. Few cells showed with cell dendrites.

The cytopathological effect of untreated UPEC endotoxins on Vero cells is shown in Fig. 3. It’s mainly effects were pathological changes with no cell lysis. The Vero cell monolayer sheet with most strains lead to lost their communication and makes the cells to be separated with shrinking. Most cells were distorted without distinction between nucleus and cytoplasm. Most of the cytoplasm was taken up by vacuolation. When the cell lysate was treated with formalin the most pathological changes disappeared.

**Discussion**

Pathogenesis is a multi-factorial process which depends on the immune status of the host, the nature of the species or strain and the number of organisms in the initial exposure. The pathogenic mechanisms of urinary tract infection have already been investigated...
in detail. UPEC strains initiate infection by binding to the superficial bladder epithelial cells that line the luminal surface of the bladder. This adherence prevents the pathogen from being washed out by the urine flow. Subsequently, bladder cells internalize the UPEC, a process that is considered an escape mechanism to protect the bacteria from the host immune system. However, internalization causes exfoliation of the superficial bladder cells harboring internalized bacteria into the urine. Before clearance, intracellular bacteria start to replicate and induce host cytokine responses, leading to the symptoms of UTI (Marquis et al., 1995; Schilling et al., 2001; Mulvey, 2002; Schmidt and Hensel, 2004). UPEC strains express a collection of strain-specific anti-host weapons called virulence markers (that include virulence factors), these markers specified bacteria to be pathogenic as mentioned later.

The five serogroups were classified toxigenic *E. coli* either O78 and O114, pathogenic *E. coli* O142, O157 or O114 and invasive *E. coli* as O164 according to the classification described by Scheutz and Stockbine (Scheutz and Stockbine, 2001). Most of them were isolated previously from urine cultures of UTI patients (Olesen et al., 1994; Gonzalez et al., 1997; Klapproth et al., 2000; Abdullah and Al-Moslih, 2005; Ananias and Yano, 2008). *E. coli* O157 which is mainly enterohaemorrhagic pathotype, isolated here from urine may be a transmission from the intestine, as in India there was a urinary Gram negative isolate, which studied by sequencing and phylogenetic analysis showed 100% similarity to reference strain *E. coli* O157 (Nandy et al., 2007). Other evidence for the suspected uropathogenicity of this strain was the sharing of 41.8% of genes not found in K12 (non-pathogenic strain) with the prototype CFT073 UPEC (Landraud et al., 2004).

Cytotoxic effect of UPEC toxin on Vero cells using different media and different treatment is a good and applicable effect study for pathogenicity extent of UPEC in vitro, which yielded a wide array of cytopathological effects worthy to be studied.

Using Syncase broth in the production of exotoxin, it was striking because of great difference and severity in cytopathological effects compared with that produced upon using nutrient broth. This may attribute to the increased production of toxins with casamino acids, which is the major constituent of syncase broth with low carbohydrate and iron. Increased toxin up to three fold with this media was proved with recombinant cholerla toxin previously (Osek et al., 1995). These toxins caused a complete Vero cell lysis, and this strong effect persists with small concentrations of toxins.

The toxins produced by UPEC here were thought to be more than one type because some of these Vero-toxins (toxic to Vero cells) was detoxified with heat while others detoxified with formalin, the last one was still effective even after formalinization, in contrast with known Vero toxin and Shiga toxin, which were inactivated by heat (Caprioli et al., 1983; Carbonell et al., 1997; Speirs et al., 1997). Vero cells cytopathic effect of treated toxin in non lysed cells presented as enlarged cells, cell membrane change and little nuclear membrane change.

Freeze-thaw lysate affects cell as a whole leading to inability to distinguishing the cytoplasm and nucleus. These may be due to cell preparation for apoptosis, vacuolation and loss of continuation between cells present may enhance this suggestion. This effect was negated after endotoxin formalinization.

In essence, the ability of pathogenic bacteria to cause disease in a susceptible host is determined by multiple virulent factors acting individually or together at different stages of infection, so bacteria equipped with virulent genes coded for these VFs. Several of these virulent genes are involved in increasing the pathogen’s fitness and adaptability.

Some researchers described the use of VFs genes content for the prediction of UTI status. Johnson et al., 2005) found that the predictors of pyelonephritis included three traditionally recognized uropathogenic traits adhesins genes (pap, afa/dra, and sfa/foc), and meningitis-associated trait (ibeA). Vranes et al. noted that the strains isolated from patients with acute pyelonephritis were found to mostly express all five or four virulence markers (O-serogroup, adhesin

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Fig. 3. Vero cell shrinkage after incubation with untreated endotoxin of UPEC isolate for 24 hours, no discrimination between nucleus and cytoplasm. 300X
type, motility, production of hemolysin, and the amount of capsular polysaccharide antigen) tested, while the less virulent strains were detected in the group of patients with chronic pyelonephritis. The lowest virulence was observed among the strains isolated in the group of patients with asymptomatic bacteriuria (Vranes et al., 2001).

To what extent can these VFs bring about the net pathological effect? We investigated VFs genes or so-called pathogenicity genes content of the isolates. It was obvious that for all isolates with a different content of VFs genes, all were produce a destructive pathogenic effect (complete cell lysis) on Vero cells equally, i.e. no significant difference between those isolates with high VFs gene content and those with low VFs gene. In vivo other conditions affect the environment of infection. As one can see, cytotoxins, secretion systems, fimbriae and others can be virulent factors in some conditions, colonization factors in other conditions and symbiosis factors in yet other conditions. Pathogenic and commensal microorganisms appear to employ similar or even identical molecular mechanisms to express their pathogenic or symbiotic potential (Hentschel et al., 2000). In particular, both pathogenic and symbiotic bacteria must actively manipulate the host immune system to make it possible for them to colonize the body. Any microorganisms will be pathogenic or commensal in a given context, under given conditions. It is the interplay between the context and the intrinsic features of a microbe that make it pathogenic or safe (Swiatczak et al., 2011). The major conclusion of our study is that pathogenic strains of UPEC can exert their pathogenic effect on living cells or systems with a few amount of virulent factors gene content in a given condition with a given context of host factors and molecules in the environment.

Disclosures

The authors have no financial conflicts of interest.

Literature


Uropathogenic *E. coli* isolates with different virulence genes


Novobiocin Sensitivity of Salmonella typhimurium dam and/or seqA Mutants

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Abstract

This study was carried out to determine the effects of novobiocin, a gyrase inhibitor, on the growth, survival, motility and whole cell proteins of S. Typhimurium dam and/or seqA strains. Our results showed that the dam and seqA/dam mutants are the most sensitive to novobiocin, compared to wild type and seqA strains. Surprisingly, the motility of seqA mutants increased after exposure to novobiocin only in stationary phase cells. All the other strains showed a significant decrease in their motility. The analysis of protein profiles of all strains demonstrated several modifications as manifested by the alteration of the expression levels of certain bands. Our work is therefore of great interest in understanding the effects of novobiocin on S. Typhimurium and the involvement of DNA methylation.

Keywords: Salmonella, DNA methylation, seqA, motility, novobiocin, proteins

Introduction

DNA methylation is a mechanism by which bacteria regulate gene expression and control several cellular processes such as transposition, DNA replication, segregation of chromosomal DNA and mismatch repair. It has been proved that Dam protein regulates gene expression and virulence of S. Typhimurium (Heithoff et al., 1999; Balbontin et al., 2006). Indeed, lack of Dam methylation disturbs the expression of std fimbriae. Heithoff et al. (Heithoff et al., 1999) have demonstrated that methylation at specific GATC sequences of promoters is crucial for transcription and alterations in the degree of methylation at promoter sequences may influence gene expression. Dam methyltransferase and regulatory proteins, such as Cap, Lrp, or OxyR compete for overlapping sites in or near promoters (Chatti and Landoulsi, 2008). Oshima et al. (2002) demonstrated that the promoters of most Dam controlled genes contained GATC sequences that overlap with recognition sites for fumarate nitrate reduction (Fnr) and catabolite activator protein (CAP) regulators. These authors suggested that the GATC network regulation takes place upstream of the coding sequences and that it is the consequence of an interaction with a regulatory protein like Fnr or CAP.

Like the Dam methyltransferase, it has been demonstrated that seqA also profoundly affects the transcription of various genes (Lobner-Olesen et al., 2003). In Escherichia coli, the product of the seqA gene is the main negative regulator of chromosome replication initiation (Lu et al., 1994, Slater et al., 1995). SeqA protein binds preferentially to hemi-methylated GATC sites and sequesters the oriC region immediately after replication (Boye et al., 1996). SeqA can also specifically bind to fully-methylated GATC sequences, not only hemimethylated, if regions other than oriC are considered. Our previous results have demonstrated that lack of seqA attenuates the virulence of S. Typhimurium in the mouse model (Chatti et al., 2007). Jakomin et al. (2008) proved that std operon is regulated by SeqA protein. The effect of SeqA on plasmid topology has been also demonstrated (Norunn and Skarstad, 1999). Indeed, it has been shown that the seqA mutation increases negative superhelicity of chromosomal and plasmid DNA (Klungsøyr and Skarstad, 2004). So, these findings may reflect a similar effect of SeqA on chromosome topology.

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It was demonstrated that novobiocin (a member of the coumermycin family of antibiotics) inhibits bacterial DNA replication and transcription. Novobiocin inhibits the DNA gyrase (Gellert et al., 1976), an enzyme that catalyzes the ATP-dependent introduction of negative superhelical turns into circular double-stranded DNA. Gyrase is needed for processes that require negatively superhelical DNA, such as DNA replication, transcription, DNA repair, and recombination (Chatterji et al., 2001). Also, it has been reported that novobiocin affects membrane integrity, nucleic acid synthesis, and cell wall synthesis (Smith and Davis, 1967).

In this study, we investigated the effects of novobiocin treatment on the survival, motility and whole cell proteins of seqA and/or dam mutants of S. Typhimurium.

Experimental

Materials and Methods

Bacterial strains and growth conditions. Bacterial strains used in this study, derived from the wild type strain S. Typhimurium 14028 are SV1610 (dam-228::MudJ) SV4752 (∆seqA1) and SV4784 (dam-225::MudJ/∆seqA1) (Jakomin et al., 2008). Overnight cultures of Salmonella were grown in nutrient broth (NB) medium and diluted into 50 ml of fresh sterile broth medium. Bacteria were routinely incubated in nutrient broth (NB) (Pronadisa, Spain) at 37°C overnight with shaking (200 rpm). Novobiocin was added at various concentrations directly to the flask and turbidity was monitored by measuring the optical density at 600 nm of the medium.

Survival study. The strains to be tested were incubated overnight at 37°C in NB. Appropriate dilutions of bacteria (10⁶ cfu/ml) were made in sterile PBS solution buffer containing various concentration of novobiocin or no drug (0, 80, 200 and 400 µg/ml) and incubated for 1 to 4 hours at room temperature. At the given time points, 100 µl of cultures were plated on Nutrient agar, after which the resulting colonies were counted and the fractional survival was calculated.

Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) determination by broth dilution. For MIC and MBC determinations, serial dilutions of novobiocin were prepared. The tubes were incubated at 37°C overnight with shaking and the highest dilution in which there were no growth was recorded as the MIC. For MBC testing, aliquots (20 µl) of broth from tubes containing no growth were plated onto solid medium and again incubated overnight at 37°C. The highest dilution in which there were no survivors was recorded as the MBC. In the above method, controls for each strain were performed using the sterile liquid medium without novobiocin. All MICs and MBCs were confirmed by triplicate assays.

Motility assays. Bacterial strains were grown in NB broth at 37°C overnight with agitation. The bacteria were then diluted 1:100 with fresh NB and incubated at 37°C with shaking. Cells were considered to be in exponential and stationary phases when they reach an optical density of 0.5 and 1, respectively. Bacterial culture (50 µl) was spotted onto an NB plate with 0.3% agar and incubated at 37°C. The concentration of novobiocin used to study the motility of all strains was 200 µg/ml. The swimming motility was estimated by measuring the diameter of the bacterial zone after incubation at 37°C during 24 hours. Media used for swarming consisted of 0.5% bacto-agar with 8 g/liter nutrient broth, to which 5 g/liter glucose was added. Swarm plates were typically allowed to dry at room temperature overnight before being used.

Whole cell proteins extraction. Pellets were resuspended in TRIS buffer (20 mM, pH 7.5) containing EDTA (5 mM) and MgCl₂ (5 mM). The cells were lysed by sonication. The cell debris was removed by centrifugation at 14 000 × g for 10 min at 4°C. The supernatant containing extracted proteins was stored at −20°C until further analysis. Protein concentration was determined according to the method of Lowry (1959).

Statistical. The experiment was repeated three times, and statistical significance was calculated using Student’s t test.

Results

Effect of novobiocin on the growth of mutants. To study the effect of the novobiocin on Salmonella growth, different concentrations ranging from zero to 400 µg/ml were tested. Compared with the control cell suspensions without novobiocin, we observed a modification of the classical cell growth curves of the different strains. Fig. 1 shows the basic phenomena observed in the inhibition of cell growth by novobiocin. The results showed that the antimicrobial activity of novobiocin increases with concentration. These two mutants do not reach the stationary phase after treatment with novobiocin at a concentration of 400 µg/ml. As the novobiocin concentration was increased, so did the duration of the lag phase whereas the rate of the growth after inhibition decreased and the cell density at which stationary phase was entered also decreased. The duration of the lag phase was proportional to the novobiocin concentration and variable according to the different Salmonella strains tested.

Effects of novobiocin on the survival of the mutants. The percentage of strains’ survival was fol-
Novobiocin sensitivity of *S. typhimurium* dam and/or *seqA*

allowed for 4 hours. Control strains were used for comparative study. Significant differences between all strains were mainly observed after treatment during 1 hour. The results obtained showed that the *dam* and *seqA/dam* strains are significantly more sensitive to novobiocin, compared to the other strains (Fig. 2). *seqA* mutant was significantly more resistant than *dam* and *seqA/dam* strains.

**Determination of MIC and MBC.** The MIC and MBC values obtained are expressed in terms of the novobiocin concentration (Table I). The MIC values shown were determined by broth dilution. MICs range from 0.6 to 1.1 mg/ml of novobiocin. The MBC values range from 2 mg/ml to 5.6 mg/ml. The double mutant *dam/seqA* presents the lowest MBC value.

**Novobiocin alters the motility of mutants.** The motility of novobiocin-treated bacteria was investigated (Fig. 3A). Statistical analysis showed significant differences in the motility of wild type, *dam* and *seqA/dam* at exponential phase after drug exposure. At stationary phase, significant difference was observed only in *dam* and *dam/seqA* strains (Fig. 3A).

**Effect of novobiocin on the whole cell proteins.** The whole cell proteins of *S. Typhimurium* and its isogenic mutants were analyzed using SDS-PAGE. When comparing the whole cell preparations on SDS-PAGE, the whole cell preparations on SDS-PAGE.

**Table I**

<table>
<thead>
<tr>
<th></th>
<th>MIC (mg/ml)</th>
<th>MBC (mg/ml)</th>
<th>MBC/MIC</th>
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<tbody>
<tr>
<td>WT</td>
<td>1.1 ± 0.11</td>
<td>5.6 ± 0.21</td>
<td>5.09</td>
</tr>
<tr>
<td><em>seqA</em></td>
<td>1.1 ± 0.14</td>
<td>5.0 ± 0.13</td>
<td>4.54</td>
</tr>
<tr>
<td><em>dam</em></td>
<td>0.6 ± 0.09</td>
<td>2.5 ± 0.13</td>
<td>4.16</td>
</tr>
<tr>
<td><em>dam/seqA</em></td>
<td>0.6 ± 0.05</td>
<td>2.0 ± 0.08</td>
<td>3.33</td>
</tr>
</tbody>
</table>

Fig. 1. Growth kinetics of *Salmonella typhimurium* and its isogenic mutants (*dam, seqA* and *dam/seqA*) under different concentrations of novobiocin: 0 µg/ml (black square), 80 (without marker), 200 µg/ml (white triangle) and 400 µg/ml (X).

Fig. 2. Novobiocin sensitivity of *Salmonella typhimurium* and its isogenic *seqA* and/or *dam* mutants. Results mentioned are the means of three repetitions. The survival assay was estimated by counting the colony forming units (CFU). The experience was monitored for 4 hours.
changes in protein profiles were observed under the two growth conditions. Therefore, as shown in Fig. 4, changes were manifested either by disappearance or modification of the expression level. In the absence of novobiocin, the alteration of protein profiles of dam and/or seqA was manifested by modification of the expression level of certain bands. After exposure to novobiocin, we noted changes of the expression level of some proteins for all tested strains. Changes of protein bands are estimated by calculating the ratio of band area before and after novobiocin treatment. Table II shows the changes observed after drug treatment.

### Discussion

It is well known that supercoiling can influence promoter activity (Wang and Lynch, 1993). Thus, DNA supercoiling affects binding of regulatory proteins, such as Dam and SeqA. In our previous work, we suggested that GATC sites could regulate virulence and stress response of *S. Typhimurium* (Chatti and Landoulsi, 2008). The growth of wild-type, *dam, seqA* and *dam*/*seqA* mutants were detected in the presence and absence of novobiocin. There was no difference in the growth rates between wild-type and *seqA* mutant strains of *S. Typhimurium* in NB medium with or without novobiocin. However, our results showed that the growth of *dam* and *seqA*/*dam* was the most sensitive mutants. Soutourina *et al.* (2001) have demonstrated that novobiocin (200 and 400 µM) induces a small decrease of the growth rate for *Pseudomonas* and *Enterobacter*. *E. coli* dam mutant was previously shown to be more sensitive to novobiocin than the wild type strain (Onogi *et al.*, 2000).

To assess the effect of the *seqA* and/or *dam* deletions on the *in vitro* susceptibility to novobiocin, we determined the MICs and MBCs as well as the loss of viable counts after exposure to 400 µg/ml of novobiocin. The MICs of novobiocin were not affected by the *seqA* inactivation but decreased in *dam* and *seqA*/*dam* strains. The viable losses in time kill experiments confirm also that the *seqA* mutant is the most resistant compared to the *dam* and *dam*/*seqA* mutants. Several studies have proved that *dam* strains are the most sensitive mutant toward many stresses such as hydrogen peroxide, bile (Chatti *et al.*, 2012; Badie *et al.*, 2007). However, all the mutants were more sensitive than the parent wild type strain. Therefore, novobiocin-induced gyrase inhibition can be expected to cause DNA strand breaks. Hence, it is not surprising that *dam* mutants, which suffer a basal level of MutHLS-induced DNA breaks, are more sensitive. Bacterial motility is a complex phenotype that is modu-

![Fig. 3. The motility of exponential (A) and stationary phase (B) cells treated (grey) and untreated (white) strains. * Significant difference (p < 0.05).](image)

![Fig. 4. The whole cell proteins of *Salmonella typhimurium* and its isogenic *dam* and/or *seqA* mutants in the absence (Column 1 to 4) or presence (Column 5 to 8) of novobiocin. Column 1 and 5: WT, Column 2 and 6: *dam*, Column 3 and 7: *seqA*, Column 4 and 8: *dam*/*seqA*. Arrows indicated the lines which show the most bands alterations.](image)

<table>
<thead>
<tr>
<th></th>
<th>L1</th>
<th>L2</th>
<th>L3</th>
<th>L4</th>
<th>L5</th>
<th>L6</th>
</tr>
</thead>
<tbody>
<tr>
<td>B1/B5</td>
<td>1.15</td>
<td>1.055</td>
<td>1.403</td>
<td>1.23</td>
<td>1.142</td>
<td>1.211</td>
</tr>
<tr>
<td>B2/B6</td>
<td>1.49</td>
<td>1.000</td>
<td>1.220</td>
<td>1.042</td>
<td>3.05</td>
<td>0.607</td>
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<td>1.003</td>
<td>1.22</td>
<td>1.098</td>
<td>3.125</td>
<td>1.064</td>
</tr>
<tr>
<td>B4/B8</td>
<td>1.31</td>
<td>1.221</td>
<td>1.33</td>
<td>1.185</td>
<td>3.21</td>
<td>1.222</td>
</tr>
</tbody>
</table>
lated by many regulators. In this study, we found that seqA mutants did not show significant defects in motility, unlike WT, dam and dam/seqA strains under this drug during exponential phase. The stationary culture of these strains demonstrated a significant increase of the motility of seqA mutant and a significant decrease in the motility of both of dam and seqA/dam mutants. However, the motility of WT strain did not change in the stationary phase. These findings suggest the implication of the nucleoid-associated protein FIS which modulate the dynamics of DNA supercoiling during the growth phase (Traversa et al., 2001). Fis promoter is activated by high negative superhelicity of the DNA in vivo and in vitro (Schneider et al., 2000). In addition, it has been proved that CRP-cAMP modulates fis expression and the inhibition of DNA gyrase represses the expression of several CRP-cAMP sensitive genes. Taken together, we can suggest that phase dependant motility especially for seqA mutant could be the consequences of a direct or indirect action of FIS and/or CRP-cAMP (Soutourina et al., 2002). The reduction in motility has been observed in E. coli in the presence of DNA gyrase inhibitors (Schneider et al., 2000). In the presence of novobiocin, a more than two fold decrease in the flihDC activity was obtained in E. coli (Soutourina et al., 2002). Also, inhibiting the DNA gyrase promotes the FimB-mediating inversion from OFF to ON and therefore it was concluded that DNA supercoiling determines the directionality of the FimB-mediated recombination (Dove and Dorman, 1994). The decrease of motility in Salmonella in the presence of novobiocin supports the link between the DNA supercoiling and motility regulation. Therefore, motility could be modulated by alteration of DNA topology, resulting from interactions between Dam or SeqA and the regulatory regions.

Whole cell proteins were investigated in the presence or absence of novobiocin. Our results showed that this drug alters the protein profiles of all strains. These changes as manifested by disappearance or modification of the expression levels of several bands. These data suggest that proteins of these strains are associated with growth and survival in the presence of novobiocin. The function of these proteins is subject to further investigation. However, changed proteins could be under the control of CRP-cAMP regulon. Further analysis by 2-DE would be needed for a better separation of proteins and more accurate estimation of sizes and to elucidate their role in bacterial response to novobiocin.

In conclusion, we suggest the involvement of DNA supercoiling on the DNA methylation control of several cellular processes. The difference in the sensitivity toward novobiocin may be due to the fatty acid composition of the membrane of each strain. Further studies on the effects of gyrase inhibitors on virulence genes expression and the deletion of gyrA and/or gyrB in seqA and/or dam mutants could help researchers to understand the relationship between DNA methylation and DNA supercoiling.

Acknowledgment

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**Literature**


The Antimicrobial Susceptibility of Helicobacter pylori Strains Isolated from Children and Adults with Primary Infection in the Lower Silesia Region, Poland

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Submitted 27 April 2013, revised 22 August 2013, accepted 16 November 2013

Abstract

The resistance of microorganisms to antibiotics has become a serious issue in recent years in the therapy of bacterial infections. This problem also concerns the treatment of infections caused by Helicobacter pylori strains. The aim of this study was to evaluate the frequency of primary resistance of H. pylori strains isolated from children and adults. The subject of the research was 105 strains of H. pylori isolated from children and 60 strains from adults in the Lower Silesia Region in the years 2008–2011. Antimicrobial susceptibility to the following antibiotics was assessed: amoxicillin (AC), clarithromycin (CH), metronidazole (MZ), tetracycline (TC), levofloxacin (LEV) and rifabutin (RB). Among the strains isolated from children, 33.3% were resistant to CH, 44.8% to MZ whereas 1.9% of strains were resistant simultaneously to CH, MZ and LEV. Among 60 strains isolated from adults, 23.3% were resistant to CH, 66.7% to MZ, and 6.7% to LEV. Moreover, 16 multidrug resistant strains were isolated from adults, including 12 resistant to CH and MZ, 3 to MZ and LEV, and 1 to CH, MZ and LEV. All examined strains were susceptible to AC, TC and RB. The high incidence of resistance to CH and MZ suggests that standard triple therapies may not be useful as first-line treatment in Poland without earlier susceptibility testing.

Key words: Helicobacter pylori, adults, children, resistance

Introduction

Colonization of the human gastric mucosa with Helicobacter pylori induces chronic gastritis and peptic ulcer disease. In addition, H. pylori plays a role in pathogenesis of gastric cancer and cancer of the mucosa-associated lymphoid tissue (Mishra, 2012). Treatment of H. pylori infection usually includes a combination of two antimicrobial agents (among amoxicillin, metronidazole and clarithromycin) and a proton pump inhibitor or bismuth salts. The ideal antimicrobial therapy should have an eradication rate of at least 90% and a low incidence of significant side effects and the drugs should be available worldwide. However, more recent publications have suggested that this level has fallen alarmingly to around 70% in many areas and even as low as 60% in some other regions (Kadayifci et al., 2006). Attempts are being made to use traditional therapeutic patterns in combination with various probiotics, e.g. Lactobacillus acidophilus (Da Silva Mendeiros et al., 2011). The success of the therapy depends on several factors, but one of the most important seems to be the increasing resistance of H. pylori strains to antibiotics (Glupczynski et al., 2001). Drug susceptibility of H. pylori strains is changeable both in different regions of the world and in different regions of the same country, so it is essential to have knowledge of the local profile of drug susceptibility of microorganisms in order to choose the most effective therapy. The aim of this study was to assess the primary resistance of H. pylori strains isolated from children and adults in the Lower Silesia Region in Poland.

Experimental

Material and Methods

The study was performed on 165 strains isolated from pediatric and adult patients of the Lower Silesia Region in the years 2008–2012. Our study involved 105 children aged 4–18 and 60 adults aged 19–89 who...
underwent endoscopic examination of the upper gastrointestinal tract due to complaints from the upper gastrointestinal tract, such as abdominal pain, nausea, or vomiting suggesting the presence of pathology. Patients had not been previously diagnosed and treated for \textit{H. pylori} infection. Patients who had previously had \textit{H. pylori} infection or received antibiotics within the last 2 months were excluded. Other exclusion criteria were parasitic diseases, allergies and autoimmune diseases. Informed written consent was obtained from each patient. The study was approved by the Bioethics Committee of Wroclaw Medical University, Approval No. 226/2011. Biopsies from the antrum and, in the case of present changes, from the corpus were taken from each patient during endoscopy of the upper gastrointestinal tract for histopathology and microbiology. Biopsies collected for microbiological examination were placed immediately after collection in sterile saline (0.15 M NaCl) and processed within two to three hours in a microbiological laboratory. The isolation and identification of strains were performed as described previously. [5] After the primary isolation and identification, the strains were kept frozen at \(-70^\circ\text{C}\) in Brucella broth containing 15% glycerol. Then the drug to six antibiotics- amoxicillin (AM), clarithromycin (CH), metronidazole (MZ), tetracycline (TC), levofloxacin (LEV) and rifabutin (RB) sensitivity – was determined by gradient diffusion (E-test, BioMerieux) with the method described by Glupczynski et al. (2001). Criteria for interpretation of results were as follows: MIC (µg/mL) for resistant strains: amoxicillin \(>0.5\), clarithromycin\(>1\), metronidazole \(>8\), tetracycline \(>1\), levofloxacin \(>0.5\) and rifabutin \(>1\) (Glupczynski et al., 2001; Megraud and Lehours, 2007).

Statistical analysis was performed by chi-square test with or without Yates’ correction and chi-square test among age groups. A p value < 0.05 was considered significant for all tests.

\section*{Results}

The total resistance of strains isolated from adults and children is shown in Table I. Among the strains isolated from children, susceptibility was indicated in 40 (38.1%) strains and 65 (61.9%) were resistant. Among the resistant strains, 35 (33.3%) were resistant to CH, including 18 (17.1%) resistant only to CH, 15 (14.3%) to CH and MZ and 2 (1.9%) to CH, MZ and LEV. Resistance to MZ was detected among 47 (44.8%) strains, 30 (28.6%) were resistant only to MZ and the others were multidrug resistant. Among 60 strains isolated from adults, 42 (70%) were resistant to at least one antibiotic, including the total resistance to CH amounting to 23.3\% (\(n = 14\)), to MZ 66.7\% (\(n = 40\)), and to LEV 6.7\% (\(n = 4\)). \textit{H. pylori} strains resistant to levofloxacin were resistant in 75\% to clarithromycin and/or metronidazole. Moreover, 16 multidrug resistant strains were isolated from adults, including 12 resistant to CH and MZ, 3 to MZ and LEV, and 1 to CH, MZ and LEV. No strains were resistant to AM, TC and RB (Table II).

In order to analyze the drug resistance of \textit{H. pylori} strains depending on age, patients were divided into 6 age groups (2–7, 8–12, 13–18, 19–44, 45–64, > 65 years) \textbf{(Fig. 1)}. The resistance of strains to clarithromycin was the highest among children aged 13–18; the percentage of strains resistant to this antibiotic decreases with age. However, in the case of metronidazole, a gradual increase of resistant strains was found, the highest percentage being noted in the group aged 45–64 (78\%). Strains resistant to levofloxacin were found among the oldest children and in the group of adults aged 45–64 (14.8\%). The percentage of strains resistant to both CH and MZ remained steady among children in all age groups (14–16\%) and adults (18.5–25\%). Strains resistant to CH, MZ and LEV were observed in the group aged 14–64 at a similar level (3.7–4.8\%).

\section*{Discussion}

In the last decade one of the most important problems of contemporary medicine has become the resistance of micro-organisms to antibiotics. This problem does not exclude \textit{H. pylori} bacilli. It is acknowledged that the appropriate course of antibiotic therapy contributes to the eradication of this micro-organism in

\begin{table}
\centering
\caption{A comparison of the frequency of primary resistance of \textit{H. pylori} isolated from children and adults.}
\begin{tabular}{|l|c|c|c|c|c|}
\hline
Resistance & Children (105) & Adults (60) & Total (165) \\
\hline
\hline
CH (total) & 35 & 33.3 & 15 & 25.0 & 50 & 30.3 \\
\hline
CH (single) & 18 & 17.1 & 0 & 0.0 & 18 & 10.9 \\
\hline
CH+MZ & 15 & 14.3 & 13 & 21.7 & 28 & 17.0 \\
\hline
CH+MZ+LEV & 2 & 1.9 & 2 & 3.3 & 4 & 2.4 \\
\hline
MZ (total) & 47 & 44.8 & 42 & 70.0 & 89 & 53.9 \\
\hline
MZ (single) & 30 & 28.6 & 24 & 40.0 & 54 & 32.7 \\
\hline
MZ+CH & 15 & 14.3 & 13 & 21.7 & 28 & 17.0 \\
\hline
MZ+CH+LEV & 2 & 1.9 & 2 & 3.3 & 4 & 2.4 \\
\hline
MZ+LEV & 0 & 0.0 & 3 & 5.0 & 3 & 1.8 \\
\hline
LEV (total) & 2 & 1.9 & 7 & 11.7 & 9 & 5.5 \\
\hline
LEV (single) & 0 & 0.0 & 2 & 3.3 & 2 & 1.2 \\
\hline
MZ+CH+LEV & 2 & 1.9 & 2 & 3.3 & 4 & 2.4 \\
\hline
MZ+LEV & 0 & 0.0 & 3 & 5.0 & 3 & 1.8 \\
\hline
\end{tabular}
\end{table}
The antimicrobial susceptibility of *H. pylori* strains

about 80% of patients (Selgrad and Malfertheiner, 2011). According to Graham and Shiotani an effective antibiotic therapy should contribute to the eradication of this micro-organism in 90–94% of patients and an excellent therapy ≥ 95% (Graham and Shiotani, 2008). The phenomenon of drug resistance of *H. pylori* strains is significant in the case of eradication therapy. In the classical therapy (IPP + AC+CH or IPP + MZ + CH) the most significant is *H. pylori* resistance to clarithromycin, which, with maintained susceptibility to MZ, lowers the effectiveness of eradication to 66% and 35% respectively (Megraud, 2004). Multicenter studies conducted in 2008–2009 by Megraud et al. concerning the primary resistance of *H. pylori* strains indicated that 31.8% of strains isolated from children and 17.5% of strains isolated from adults were resistant to clarithromycin. However, the resistance of strains to metronidazole was 25.7% and 34.9% respectively (Megraud et al., 2013). Our studies have indicated high resistance to clarithromycin among children (33%) and adults (25%). The resistance of examined strains to metronidazole in both groups was considerably higher (average 53.9%). Since the mid-1990s, the gradual increase of *H. pylori* primary resistance to applied antibiotics has been observed. In Poland, the percentage of strains resistant to clarithromycin varies depending on the region, from 21% to 28% (Karczewska et al., 2011, Dzierżanowska-Fangrat et al., 2005, Andrzejewska et al., 2009). A diverse level of resistance to clarithromycin was noted in other countries (5.6–36%). The resistance rates in Europe (11.1%),

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Resistant strains</th>
<th>Range</th>
<th>MIC&lt;sub&gt;50&lt;/sub&gt;</th>
<th>MIC&lt;sub&gt;90&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH</td>
<td>50</td>
<td>30.3 (26.9–33.7)</td>
<td>0.016</td>
<td>1.5</td>
</tr>
<tr>
<td>MZ</td>
<td>89</td>
<td>53.9 (44.41–63.47)</td>
<td>16</td>
<td>126</td>
</tr>
<tr>
<td>LEV</td>
<td>9</td>
<td>5.5 (3.69–7.22)</td>
<td>0.064</td>
<td>0.125</td>
</tr>
<tr>
<td>AM</td>
<td>0</td>
<td>0 (0.0–0.0)</td>
<td>0.016</td>
<td>0.016</td>
</tr>
<tr>
<td>RB</td>
<td>0</td>
<td>0 (0.0–0.0)</td>
<td>0.02</td>
<td>0.02</td>
</tr>
<tr>
<td>TC</td>
<td>0</td>
<td>0 (0.00–0.01)</td>
<td>0.023</td>
<td>0.047</td>
</tr>
</tbody>
</table>

No – number, CH – clarithromycin, MZ – metronidazole, LEV – levofloxacin, AM – amoxicillin, TC – tetracycline, RB – rifabutin, MIC<sub>50</sub> – Minimum Inhibitory Concentration required to inhibit the growth of 50% of organisms, MIC<sub>90</sub> – Minimum Inhibitory Concentration required to inhibit the growth of 90% of organisms

![Fig. 1. Resistance of *H. pylori* strains in different age groups.](image-url)
Asia (18.9%) and America (29.3%) significantly differ (De Franceso et al., 2010). In Europe we can observe large differences in resistance of strains to clarithromycin between northern (10.0%) and southern Europe (20.0%) (Megraud et al., 2013).

Ten years ago, in Poland, the level of strains resistance to metronidazole was in the range 27–52% (average 37%) (Dzierżanowska-Fangrat et al., 2002) and regional variation was observed. Children from the Lower Silesia region have the highest noted resistance, at 52%. A lower percentage of resistant strains could be observed in children from Warsaw (37%), Wielkopolska Region (45%), Kujawsko-Pomorskie Region (20%) and from Łódzki Region (18%) (Bąk-Romaniszyn et al., 2004; Łazewicz, 2003). The frequency of primary resistance to metronidazole among adults in Europe fluctuated between 28% and 44%. No significant differences were found between southern and northern parts of the continent (respectively ~ 29.7% and 28.6%). It was noted, however, that the resistance of strains is considerably higher in western and in central Europe (43.8%) (Megraud et al., 2013). In connection with more frequent usage of both clarithromycin and metronidazole in *H. pylori* eradication therapy, the steady increase of strains resistant to two antibiotics at the same time was noted. Towards the end of the 1990s, such resistance was low in Europe and amounted to only 0.8–9.1% (Boyanova, 2009; Elviss et al., 2004). In 2009, in Spain 17.2% of strains were noted to be resistant to both clarithromycin and metronidazole among children (Aqudo et al., 2009). In our study, the percentage of strains primarily resistant to clarithromycin and metronidazole was 14% among children and 21.7% among adults (average 17%). Similar results were obtained in other regions of Poland (13–15.5%) (Dzierżanowska-Fangrat et al., 2005).

The growth tendency also shows the resistance of strains to levofloxacin and currently in Europe, from 7% to 13% of *H. pylori* strains were noted to be resistant. In southern Poland, this resistance level is 12% (Karczewska et al., 2012; Megraud et al., 2013). In our study total primary resistance to LEV was 5.5%, including resistance in children (1.9%) and in adults (11.7%). Moreover, alarmingly, in our region we have observed 1.9% of strains resistant among children and 3.3% among adults to three antibiotics simultaneously. Multidrug-resistant *H. pylori* strains were also observed in other countries, such as Italy, Bulgaria and Taiwan (Boyanova and Mitov, 2010; Yang et al., 2010).

In this work no resistance of *H. pylori* strains to amoxicillin, tetracycline and rifabutin was noted. Other authors also have not found resistance to these antibiotics (Megraud et al., 2013; Karczewska et al., 2011; Gościniak et al., 2004). Primary amoxicillin resistance has been low (< 2%) in Europe but higher (6–59%) in Africa, Asia and South America (Boyanova and Mitov, 2010; Yang et al., 2010). Resistance to tetracycline is low (< 3%) all over the world, except for Africa, where it amounts to 44% (De Franceso et al., 2010). The observed low resistance to rifabutin so far is very low, 1.3%, and the effectiveness of therapy with rifabutin is good, 73%. Treatment of *H. pylori* infections with rifabutin is promising, especially after failures of eradication with standard antibiotics such as: amoxicillin, metronidazole, clarithromycin, levofloxacin, and tetracycline (Gisbert and Calvet, 2012). However, it should be remembered that the low resistance of *H. pylori* to rifabutin arises from using this antibiotic only to treat *Mycobacterium tuberculosis* (Nishizawa et al., 2011). In conclusion, high incidence of resistance to CH and MZ suggests that standard triple therapies may not be useful as first-line treatment in Poland without earlier susceptibility testing. The use of antibiotics for other indications seems to be the major risk factor for development of primary resistance.

The authors declare no conflict of interest.

**Literature**


The antimicrobial susceptibility of *H. pylori* strains


**Helicobacter pylori Infection in Type 1 Diabetes Children and Adolescents Using 13C Urea Breath Test**

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Submitted 8 August 2013, revised 31 October 2013, accepted 16 November 2013

**Abstract**

There is a 10–30% prevalence of HP infection in the general pediatric population in Poland. This study aimed to determine its prevalence in T1DM children in Upper Silesia, Poland and estimate its influence on metabolic control of patients. We studied 149 (82♀) children with T1DM (duration > 12 months, mean HbA1c) and 298 (164♀) age-matched controls. In all cases height and weight z-scores and Cole’s index were assessed. In T1DM patients additionally glycated hemoglobin A1c and T1DM duration were analyzed. Presence of HP infection was determined using 13C-isotope-labeled urea breath test (UBT) (fasting and 30min after ingestion 75 mg of 13C urea). HP infection was present in 17 (11.4%) T1DM patients and in 49 (16.4%) controls (p > 0.05). T1DM patients presented higher values of anthropometric parameters than healthy controls (weight SDS 0.25 [–0.46÷0.84] vs. –0.25 [–1.06÷0.26], height SDS 0.09 [–0.60÷0.69] vs. –0.31 [–1.17÷0.48] and Cole’s index 103% [93÷111]% vs. 97% [86÷106%]; for all p < 0001). Within both groups – T1DM children and controls – no differences regarding sex, age and any of the anthropometric parameters were determined. T1DM duration and HbA1c showed no relation to prevalence of HP infection. Prevalence of HP infection in pediatric T1DM patients is similar to that of healthy peers and shows no relation to glycemic control.

**Key words:** Helicobacter pylori, HbA1c, type 1 diabetes, urea breath test

**Introduction**

Helicobacter pylori (HP) causes one of the most common chronic infections worldwide. This is a gram-negative, spiral-shaped pathogenic bacterium that specifically colonizes the gastric epithelium and is related to chronic gastritis and duodenitis, peptic ulcer disease and functional gastrointestinal motor disorders (Marshall and Warren, 1984; Wotherspoon et al., 1991). The remote consequences of a chronic infection, which appear especially in adulthood, include: peptic ulcer, mucosa-associated lymphoid tissue (MALT) lymphoma and gastric cancer.

The prevalence of HP infection varies between countries and depends on the age and socio-economic status. It is estimated that in developed European countries 10–30% of the population is infected, however most cases remain asymptomatic. In developing countries the prevalence in the adult population raises even up to 70% (Goh et al., 2011). In previous research the prevalence of HP infection in the general pediatric population in Poland was assessed to be 30% (Iwanczak et al., 2004). Nowadays the frequency of infection seems to be lower than formerly reported. An assessment by Żabka et al. (2010) revealed a 10% prevalence and a most recent publication showed that the frequency of HP infection in healthy children in Poland was 15.7% (Krusiec-Świdergol et al., 2010).

It is known that patients with diabetes are more prone to infection than healthy people. This is probably because of the fact that both cellular and humoral immune systems are perturbed, especially in the context of poor glycemic control with severe complications (de Luis et al., 1998; Mustapha et al., 2005). Many studies have evaluated the prevalence of the HP infection in diabetic patients, but the epidemiological relationship remains controversial (Ariizumi et al., 2008; Ciortescu et al., 2009; Ojetti et al., 2001, 2010; Otto-Buczkowska, ...
2012; Stępkowski and Grzeszczyk, 2009). Furthermore, publications investigating HP infection in children and adolescents with type 1 diabetes (T1DM) are sparse and their conclusions contradictory (Arslan et al., 2000; Colombo et al., 2002; Khalil et al., 2007; Ojetti et al., 2010; Toporowska-Kowalska et al., 2006, 2007). Some researchers revealed higher prevalence of HP infection in diabetic patients in general as well as in the pediatric population with T1DM (Ariizumi et al., 2008; Arslan et al., 2000; Ciortescu et al., 2009; de Luis et al., 1998; Ojetti et al., 2001, 2010; Otto-Buczkowska, 2012; Stępkowski and Grzeszczyk, 2009). Nevertheless, the results of other authors have not confirmed such observations (Ciortescu et al., 2009; Colombo et al., 2002; Khalil et al., 2007; Ojetti et al., 2001; Toporowska-Kowalska et al., 2006, 2007).

The studies in T1DM subjects yielded also additional data on the relationship between HP infection and glycemic control, although their conclusions are also not unequivocal. On one hand, there are reports of a negative impact of HP infection on HbA1c levels (Toporowska-Kowalska et al., 2006, 2007), but discordant conclusions have also been published (Candelli et al., 2003; Colombo et al., 2002; Khalil et al., 2007). A supplementary interesting assessment revealed that the eradication of HP infection did not improve the metabolic control in a short-term follow-up (Candelli et al., 2004, 2012; Khalil et al., 2007). Although a study by Begue et al. (1999) demonstrated that HP eradication might decrease HbA1c.

The aim of the present study was to determine the prevalence of HP infection in children and adolescents with T1DM in Upper Silesia, Poland and to estimate the influence of HP presence on metabolic control of the patients.

**Experimental**

**Material and Methods**

**Patients.** The study group comprised of 149 (82 female and 67 male) T1DM children and adolescents, aged 13.4 ± 3.4 years. Volunteers from the regional out-patient diabetes clinic were enrolled using the following criteria: DM duration > 12 months, no history of antibiotic or proton pump inhibitor therapy in the past 4 weeks. The patients were examined between July 2011 and June 2012. Table I. shows the detailed characteristics of the studied patients. The control group consisted of 298 age- and sex-matched children and adolescents (164 female and 134 male) with no known glucose metabolism disorders – characteristics are presented in Table I. This group was enrolled from a large cohort (n = 1253) of children investigated during the program “Good diagnosis-Treatment-Live” carried out at the Clinical Hospital No 1 in Zabrze, Poland between March 2010 and June 2012.

**Methods.** In all T1DM patients Z-score for height and weight, Cole’s index and glycated hemoglobin A1c (HbA1c) were assessed. Height and weight in T1DM patients were measured using a standardized scale and the Martin stadiometer. HbA1c measurements were carried out in the same laboratory using the recommended high pressure liquid chromatography (HPLC), which is a DCCT reference method. The normal range for this laboratory is < 6%.

The presence of HP infection was determined in all subjects by means of a breath test (UBT) with 13C isotope-labeled urea, which was performed fasting. Breath samples – at baseline and 30 minutes after ingestion of 75 mg of 13C isotope-labeled urea – were collected in 650 ml aluminized bags with one-way valves. An infrared spectrophotometer (IRIS, Wagner GMBH, Germany) was used to measure the 12CO2/13CO2 ratio. The results of the test were considered positive if C13 concentration (delta over baseline, DOB [o/oo]) raised in the exhaled air by more than 4.0‰ (Żabka et al., 2010).

**Statistical analysis.** Statistical analysis was performed with the R software (www.bioconductor.org). Descriptive statistics were calculated for all analyzed parameters, and adequate figures were generated. To detect the outlying values Tukey’s criterion was used. To check distribution normality Lilliefors test was employed. The verification of the variance homogeneity hypothesis was carried out using the F or Bartlett statistic. ANOVA algorithm and Student’s t test were performed for comparative analysis of normally distributed variables. In the case of a distribution other than normal, we used the non-parametric ANOVA

<table>
<thead>
<tr>
<th>UBT result</th>
<th>T1DM Patients</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>Total 149</td>
<td>Total 298</td>
</tr>
<tr>
<td>Age [yrs]</td>
<td>13.4 ± 3.4</td>
<td>13.3 ± 3.3</td>
</tr>
<tr>
<td>Weight Z-score</td>
<td>0.25 ± 0.93</td>
<td>-0.25 ± 1.07</td>
</tr>
<tr>
<td>[–0.46 ÷ 0.84]</td>
<td>[–1.06 ÷ 0.26]</td>
<td></td>
</tr>
<tr>
<td>Height Z-score</td>
<td>0.09 ± 0.97</td>
<td>-0.31 ± 1.23</td>
</tr>
<tr>
<td>[–0.60 ÷ 0.69]</td>
<td>[–1.17 ÷ 0.48]</td>
<td></td>
</tr>
<tr>
<td>Cole’s index [%]</td>
<td>103 ± 13</td>
<td>97 ± 15</td>
</tr>
<tr>
<td>[93 ÷ 111]</td>
<td>[86 ÷ 106]</td>
<td></td>
</tr>
<tr>
<td>T1DM Duration [yrs]</td>
<td>4.6 ± 3.5</td>
<td></td>
</tr>
<tr>
<td>HbA1c [%]</td>
<td>7.69 ± 1.63</td>
<td></td>
</tr>
</tbody>
</table>

Legend: N – number of children, ± SD, [±95% CI], SDS – standard deviation score, T1DM – Type 1 diabetes mellitus, UBT – urea breath test, HbA1c – hemoglobin A1c.
positive UBT result was observed in 49 (16.4%) cases. Similar as in the T1DM group no differences regarding sex, age and no relation to the investigated anthropometric parameters were found (Table II).

To investigate the discrepancy, all anthropometric parameters – height, weight, Cole’s index – were determined. Parameters associated to T1DM – disease duration as well as HbA1c – showed no relation to the prevalence of HP infection (Table II). Within the control group a positive UBT result was obtained in 49 (16.4%) cases. Similar as in the T1DM group no differences regarding sex, age and no relation to the investigated anthropometric parameters were found (Table II).

The comparison of the frequency of HP infection in T1DM patients and non-diabetic children revealed no discrepancy. All anthropometric parameters – height and weight z-scores as well as the Cole’s index – were significantly higher in T1DM patients (Table I).

### Discussion

This study was performed using the UBT as a gold standard method (Koletzko et al., 2011). It was conducted in a large number of T1DM pediatric patients presenting quite good metabolic control (HbA1c 7.69%). The observed frequency of HP infection among the non-diabetic cohort (approximately 16%) was similar to the newest reports regarding the general polish pediatric population, which demonstrated a decrease of the prevalence in comparison to the past decade (Iwańczak et al., 2004; Krusiec-Swidergoł et al., 2010; Żabka et al., 2010).

The results of former studies investigating HP infection in patients with T1DM are contradictory and there are only few publications concerning the pediatric population (Arslan et al., 2000; Ciortescu et al., 2009; Colombo et al., 2002; de Luis et al., 1998; Ojetti et al., 2001; Toporowska-Kowalska et al., 2006, 2007). Some of them revealed a higher prevalence of HP infection in children with T1DM (Arslan et al., 2000; de Luis et al., 1998). However, our findings and the results of other authors have not confirmed such observations (Candelli et al., 2003; Ciortescu et al., 2009; Colombo et al., 2002; Khalil et al., 2007; Ojetti et al., 2001; Toporowska-Kowalska et al., 2006, 2007). Given the facts mentioned in the former paragraph it seems justified to make an assumption that the prevalence of HP infection in T1DM children is the same as in healthy peers. The frequency of HP infection in the studied patients is lower than in earlier publications (Candelli et al., 2003; Colombo et al., 2002; Toporowska-Kowalska et al., 2006, 2007). This might be explained by the fact that some of the studies were based on a serological screening that does not distinguish past and present infections.
as well as by the general decrease of HP infection in Polish and European children during the last decade (Colombo et al., 2002; Iwańczak et al., 2004; Krusiec-Swidergol et al., 2010; Żabka et al., 2010).

In our study T1DM children with a confirmed HP infection did not differ significantly in terms of metabolic control and disease duration. No association between HP infection and HbA1c was also found by other authors (Candelli et al., 2003; Colombo et al., 2002; Khalil et al., 2007). In addition HP eradication was shown to have no impact on metabolic control (Candelli et al., 2004, 2012; Khalil et al., 2007). However, in the former study in T1DM polish children, those infected with HP presented higher HbA1c levels (Toporowska-Kowalska et al., 2007). Also Begue et al. (1999) suggested a positive relation between HP eradication and metabolic control. These discrepancies might be related to the differences between the study groups, especially regarding age, race and socioeconomic status. Moreover, the link between HP infection and metabolic control might be indirect (i.e. HP is known to influence the gastric motility and was shown to affect insulin resistance and may in this way influence blood glucose and HbA1c levels) and therefore difficult to determine clearly (Gen et al., 2010; Toporowska-Kowalska et al., 2006). The relation between HP infection and diabetes duration was investigated only by few other researchers, but all findings are similar to our results (Candelli et al., 2003; Colombo et al., 2002; Khalil et al., 2007; Toporowska-Kowalska et al., 2006, 2007).

Epidemiological studies suggest that the prevalence of HP infection should rise with age (Krusiec-Swidergol et al., 2010). Despite that fact research conducted in T1DM children showed no significant relation between HP infection and age (Khalil et al., 2007; Toporowska-Kowalska et al., 2006, 2007). Our results revealed also no dissimilarities in age between HP positive and negative children in both groups – T1DM and controls.

Because HP was in the past described to potentially impair growth in the pediatric population, our study also assessed the standardized anthropometric parameters of T1DM patients and non-diabetic controls (Krusiec-Swidergol et al., 2010). All mean values for both groups remained well within the normal ranges (90–110% for Cole’s index and –1.0–1.0 for Z-scores), although both cohorts differed significantly by all of the anthropometric measurements. As expected T1DM children had slightly higher mean weight and height Z-scores as well as a little higher mean Cole’s index. Nonetheless, within both groups HP positive and negative children presented no discrepancies regarding the analyzed anthropometric parameters.

The strong aspects of this study are – except for the ones mentioned in the beginning of the discussion – the race and ethnic homogeneity of the studied groups. Nonetheless, because patients were not randomly chosen for this study, the presented data cannot be considered to represent the incidence of HP infection in the whole pediatric T1DM population in Poland.

The observed prevalence of HP infection in children and adolescents with T1DM is similar to that of healthy peers. No evidence for a relationship between the glycemic control and presence of HP infection in patients with T1DM could be determined.

Acknowledgements

This study was partially financed by the grant SUT-BK/RAU1/2013/10. Agata Chobot received partial financing by MNiSW IP2012 007672.

Literature


Introduction

Enteroviruses (EVs) are members of the Picornaviridae family, a large and diverse group of small RNA viruses characterized by a single-positive-strand genomic RNA. They affect millions of people worldwide each year, and are often found in the respiratory secretions and stool of an infected person. Infection can result in a wide variety of symptoms ranging from mild respiratory illness (common cold), through hand, foot, and mouth disease, acute haemorrhagic conjunctivitis, aseptic meningitis, and myocarditis, to severe neonatal sepsis-like disease and acute flaccid paralysis. (Tapparel, 2012)

The classic method for diagnosis of infection with EVs has been virus isolation by cell culture from stool samples, throat swabs, or cerebrospinal fluid (CSF). Cell culture is still the best method for determining the occurrence of infectious viruses in environment, as well. Viral isolation is possible in a variety of cell lines; however, no single cell line is optimal for all EVs.

PCR methods for detecting enteroviruses have been developed during the past decade. Many studies have shown that PCR is more sensitive and rapid than virus isolation for the diagnosis of enteroviral infection. Although detection by PCR reveals the presence of viral RNA, it does not indicate the infectivity of the virus.

The determination of viral infectivity is important especially in environmental monitoring. The detected genomic material may be present in otherwise defective virus particles that are not able to bind to or replicate in the host cells. Knowing if a virus is infectious is important from a public health perspective to determine if there is a public health concern. The infectivity of viruses may be determined with the use of cell cultures, such as Caco-2 and RD (Terletskaya-Ladwig et al., 2008; Sedmak et al., 2005). Caco-2 cell line is a continuous line of heterogeneous human epithelial colorectal adenocarcinoma cells. It has been found that Caco-2 cells support the growth of many viruses, including enteroviruses, rotaviruses, adenoviruses, and astroviruses (Hamza 2011; Pinto et al., 1995). However, WHO recommended RD cells for isolation of polioviruses and other enteroviruses. Most of the enterovirus serotypes have been propagated in RD cells, but in practice isolation from clinical and environmental material is often unsuccessful (Lipson et al., 1988; Witek et al., 2011).

The present study was conducted to obtain information about the utility of Caco-2 cell in the isolation of enteroviruses from environmental (sewage) and clinical samples. CSF is not a simple material for enterovirus strains isolation, because the enterovirus load is lower than in faeces during enteroviral meningitis, and also is rich in replication-defective particles. Sewage is
a heterogeneous material rich in factors such as metals, humic acids, and other organic matter which can be toxic for cells. Our goal was to compare EVs’ isolation from this material in Caco-2 and RD cells.

**Experimental**

**Materials and Methods**

**CSF samples.** Cerebrospinal fluid samples were obtained in 2011 and 2012 from patients with suspicion of enteroviral infection, and sent to the Laboratory of Virology in the National Institute of Health for viral diagnostics. Thirty-two samples of CSF positive in Pan-entero RT-PCR were taken for EV strain isolation in cell culture. These samples were divided into two groups: strong positive – with intensive band after RT-PCR reaction and weak positive – with not clearly seen band. A volume of 200 µl of cerebrospinal fluid was inoculated into tubes with RD and Caco-2 cells. The tubes were incubated at 37°C. Each specimen underwent two passages in RD and Caco-2 cells. Samples demonstrating viral cytopathic effect (CPE) were identified by neutralization assay using specific antisera (National Institute of Public Health and the Environment, the Netherlands).

**Sewage samples.** Samples of raw sewage were processed according to the protocol described earlier (Zurbriggen et al., 2008). Tubes with monolayer of RD and Caco-2 cells in maintenance medium were inoculated with 100 µl of sewage sample; before inoculation, samples were incubated with different concentrations of trypsin (from 0 to 50 µg/ml) for different times (from 0 min to 2 h) at 37°C and next were added to those tubes. Cytopathic effects were read daily for 7 days, and two passages were performed. Supernatant fluids from samples demonstrating viral CPE were used for the RT-PCR detection to confirm the presence of the virus. Four independent experiments were performed.

**RNA extraction and RT-PCR.** Viral RNA was extracted from 140 µl of CSF, cell culture supernatant or concentrated sewage using spin columns (Qiagen) following the manufacturer’s instructions. RT-PCR was carried out using Pan-enterovirus primers for enterovirus detection based on the WHO manual (WHO, 2004). This set of primers produces a product of 114 bp and has been designed to detect and amplify a genome segment present at the 5’-UTR of the enterovirus genomes. RT-PCR amplification was performed: one cycle of reverse transcription at 45°C for 20 min; one cycle of denaturation at 94°C for 2 min; 30 cycles of denaturation at 94°C for 30 s; annealing at 55°C for 30 s; elongation at 70°C for 30 s followed by one cycle of elongation at 70°C for 7 min. Reaction mixtures were then held at 4°C. Amplification products were analysed in 2% agarose gels, GelRed-stained, and examined under a UV DNA trans-illuminator.

**Statistical analysis.** Received data were analysed with Statgraphics for Windows, Centurion, v.XV. StatPointTech.Inc. USA. Simple linear regression and multiple regression model was used to estimate relationships among variables.

**Results**

A total of 177 cerebrospinal fluid samples were obtained in 2011 (59) and 2012 (118) from patients with suspicion of enteroviral infection, and sent to the Laboratory of Virology in the National Institute of Health for viral diagnosis. Of 177 CSF samples, 85 (48%) samples were positive in RT-PCR reaction (36% in 2011 and 60% in 2012). Thirty-two CSF samples were selected from all positive samples. These samples were divided into two groups: strong positive (16) – with intensive band after RT-PCR reaction and weak positive (16) – with not clearly seen band. A volume of 200 µl of cerebrospinal fluid was inoculated into tubes with RD and Caco-2 cells according to WHO procedures. Out of the 32 samples analysed, 22 (68.75%) were positive for enteroviruses by isolation in Caco-2 cells, and 10 (31.25%) were positive by isolation in RD cells (Table I). There were two positive isolations in RD cells (12.5%) in the group of weak-positive samples unlike isolation in Caco-2 cells – 50.0% (8) positive results. The best results were obtained in the group of strong-positive samples (intensive band) isolated in Caco-2 cells – 87.5% positive results. However, positive isolation from this group of material in RD cells was much lower (50.0%). Serotyping revealed the predominance of echovirus 6 (E6), followed by E11.

Samples of sewage were collected from several locations around Poland in 2011. Samples were evaluated
The utility of Caco-2 cells in isolation of enteroviruses

1 by RT-PCR assay for the presence of enteroviruses. Positive samples were pooled and isolated in Caco-2 and RD cells. Before inoculation, sewage samples were incubated with different concentrations of trypsin (from 0 to 50 μg/ml) for different times (from 0 min to 2 h) at 37°C. Among 80 samples isolated in RD cells, only 2 were positive (Table II) in contrast to 43 positive samples in Caco-2 cells (Table III). The probability of isolation in RD cells (P = 0.025) was 20 times lower that in Caco-2 cells (P = 0.538). The processing of samples before inoculation (different concentrations of trypsin and different incubation times) did not significantly influence on the isolation of enterovirus strains in RD and Caco-2 cells. There was no significant difference between incubation time and concentration of trypsin and positive results of isolation.

All samples demonstrating characteristic cytopathic effect were positive in Pan-enterovirus RT-PCR. Thus, it confirmed that observing cytopathic effect was connected with the presence of enterovirus.

Discussion

There is currently no cell line that supports the growth of all enterovirus serotypes. Different cell lines such as HEL, Caco-2, RD, HEP2, A549, and buffalo green monkey kidney cells (BGMK) are used for isolation of enteroviruses (Schmidt et al., 1975; Kok et al., 1998; Buxbaum et al., 2001; Otero et al., 2001; Buck et al., 2002). In this study, Caco-2 cell line was used for isolation of enteroviruses from sewage and cerebrospinal samples. In addition to Caco-2 cells, samples were also evaluated by RT-PCR assay for the presence of enteroviruses, and isolated in RD cells. Caco-2 cells were more effective in enterovirus isolation than RD cells. The rates of enterovirus isolation for 32 samples of cerebrospinal fluid positive after PCR reaction resulted in rate increase for isolation in Caco-2 cells and RD cells (87.5% and 50%, respectively).

Table II

Sewage samples analysis for enteroviruses in RD cells.

<table>
<thead>
<tr>
<th>Trypsin concentration (μg/ml)</th>
<th>Incubation time</th>
<th>No of positive results</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0'</td>
<td>10'</td>
</tr>
<tr>
<td>0</td>
<td>0000</td>
<td>0000</td>
</tr>
<tr>
<td>P = 0</td>
<td>P = 0</td>
<td>P = 0</td>
</tr>
<tr>
<td>5</td>
<td>0000</td>
<td>0000</td>
</tr>
<tr>
<td>P = 0</td>
<td>P = 0</td>
<td>P = 0</td>
</tr>
<tr>
<td>10</td>
<td>0000</td>
<td>0000</td>
</tr>
<tr>
<td>P = 0</td>
<td>P = 0</td>
<td>P = 0</td>
</tr>
<tr>
<td>50</td>
<td>0000</td>
<td>+000</td>
</tr>
<tr>
<td>P = 0</td>
<td>P = 0.25</td>
<td>P = 0</td>
</tr>
</tbody>
</table>

Table III

Sewage samples analysis for enteroviruses in Caco-2 cells.

<table>
<thead>
<tr>
<th>Trypsin concentration (μg/ml)</th>
<th>Incubation time</th>
<th>No of positive results</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0'</td>
<td>10'</td>
</tr>
<tr>
<td>0</td>
<td>++++0</td>
<td>++++0</td>
</tr>
<tr>
<td>P = 0.75</td>
<td>P = 0.75</td>
<td>P = 0.75</td>
</tr>
<tr>
<td>5</td>
<td>+000</td>
<td>++++0</td>
</tr>
<tr>
<td>P = 0.25</td>
<td>P = 0.75</td>
<td>P = 0.50</td>
</tr>
<tr>
<td>10</td>
<td>++++0</td>
<td>++++0</td>
</tr>
<tr>
<td>P = 0.75</td>
<td>P = 0.75</td>
<td>P = 0.75</td>
</tr>
<tr>
<td>50</td>
<td>+000</td>
<td>+000</td>
</tr>
<tr>
<td>P = 0.25</td>
<td>P = 0.25</td>
<td>P = 0.75</td>
</tr>
</tbody>
</table>

P – probability; 0 – lack of cytopathic effect; + – characteristic enterovirus cytopathic effect.
Also, the probability of isolation of enteroviruses from sewage in Caco-2 cells was 20 times higher that in RD cells. All positive isolations in Caco-2 cells were observed in the first passage; it was impossible in RD cells. In the case of RD cells, all positive isolations were observed in the second passage. The cell culture of Caco-2 cells after inoculation was in good condition for several days in contrast to RD cell culture. RD cells showed rapid degeneration due to non-specific toxicity of the specimen (sewage). Negative results of isolation in RD cells are probably a consequence of the presence of compounds that are toxic for cells. The presence of factors such as metals, humic acids, and other organic matter can interfere with cell growth. Toxic or inhibitory compounds which can impair the sensitivity of cell culture and molecular systems are found frequently in environmental samples (Murrin and Slade 1997; Reynolds et al., 1996). Toxicity in cell culture can be variable; excessive toxicity causes widespread cell death while limited toxicity may adversely affect virus attachment to cells.

It is interesting to note that in a study by Reigel (1985), different viruses from clinical material replicated in Caco-2 cells: enteroviruses (coxackieviruses B1-B6, poliovirus types 1-3, most echoviruses and coxsackieviruses A), adenoviruses, herpes simplex virus types 1 and 2, measles viruses, respiratory syncytial viruses, parainfluenza type 2 viruses, and to a lesser extent rubella and mumps viruses. However, Caco-2 cells are not popular in environmental analysis. Our study demonstrates that the resistance of Caco-2 cells for cytotoxic components from sewage is of crucial importance. It should also be considered that in years with high activity of types growing well in cell culture, the sensitivity of cell culture can be higher than in years with lower enterovirus activity (Roth et al., 2007).

The addition of trypsin does not affect enterovirus isolation. The proteolytic enzymes have been reported to have several effects on cell cultures and virus cultivation. Enzyme treatment enhanced infectivity in most enteric viruses, for example in reoviruses (Rutjes et al., 2009). Trypsin treatment is not recommended in enterovirus isolation. That enzyme can cleave some enterovirus capsid protein, but usually does not affect infectivity. The high probability of enteroviruses’ isolation in Caco-2 cells in our study demonstrates that sewage testing may be very useful in epidemiological study of enteroviruses circulating in the population. Sewage surveillance system has been shown to be more sensitive than reporting of clinical cases of serious illness in a community (Sinclair et al., 2008; Bosch et al., 2008). Data regarding the occurrence of viruses in raw sewage may provide an overview of the epidemiology of virus infections circulating in the community, and at the same time may reveal the occurrence of asymptomatic infections (Pinto et al., 2007; Lodder et al., 2012). The usefulness of community sewage testing to monitor the presence of polioviruses in the face of the circulation of wild-type poliovirus in the community has been demonstrated in the Netherlands (Van der Avoort et al., 1995) and Finland (Poyry et al., 1988), and similar testing of sewage may be useful for the monitoring of echoviruses and coxsackieviruses.

In conclusion, using Caco-2 cells for virus isolation in sewage seems to be very promising for environmental surveillance of enterovirus circulation and epidemiology, with all the significant effects that this may have on public health. Therefore, it is always important to consider the limitations imposed by the toxicity of environmental samples when selecting cells for viral isolation. Also, the isolation of enterovirus strains from clinical material (e.g. cerebrospinal fluids) with the use of Caco-2 cells seems to be very promising.

Acknowledgments
This study was supported by Ministry of Science and Higher Education of Poland grant NN 404 113 839.

Literature


The utility of Caco-2 cells in isolation of enteroviruses


Polar ecosystems are extensively prospected for obligate psychrophilic and psychrotrophic microbes in order to discover the unique adaptabilities of their enzymes (de Pascale et al., 2012). It is evident that these ongoing research have yield fruitful discoveries of varieties of microbial communities capable of producing cold-active enzymes. In the Arctic region, several bio-prospecting studies had led to the findings of a diverse range of bacteria capable of producing cold-active enzymes. A study of bacterial diversity from sediment samples along a transect from the snout of the Arctic Midtre Lovenbreen glacier up to the melt water streams uncovered 32 groups of bacteria, with 14 exhibiting cold-active enzyme activities (Reddy et al., 2009). Sea ice that provides a large pool of organic matters housed several bacterial communities capable of producing cold-active hydrolytic enzymes. Arctic sea ice samples from Canada Basin yield 338 bacterial strains having diverse enzyme producing capabilities, with lipase being the highest (Yu et al. 2009). Sea ice and seawater sampled from Spitzbergen fjords, possessed 116 psychrophilic and psychrotrophic strains; with protease enzyme producers accounted for more than half of the isolated bacteria (Groudieva et al., 2004). A total of 103 bacterial isolates from 47 phylotypes were isolated from eight sediment and one soil samples of two Arctic fjords of Kongsfjorden and Ny-Ålesund, Svalbard with 56% showing either amylase or lipase or both activities (Srinivas et al., 2009). The Finnish Lapland exhibited large annual temperature variations and freeze/thaw events as compared to other Arctic areas. Various ecosystems of Finnish Lapland sampled in Männistö and Häggblom (2006) revealed 331 bacterial strains with protease and lipase producers detected in 59 and 66 isolates.

Extensive research on cold-loving microorganism was carried out because there is a growing prospect of cold-adapted enzymes in the industries. These enzymes were functional at varied and demanding
industrial conditions. Cold adapted enzymes are in favor because of their ability to save energy, reduce mesophilic microbial contamination and the ease of mild heat-inactivation to terminate the cold-adapted enzymes catalysis (Groudieva et al., 2004). Lipase catalyze both the hydrolysis and synthesis of ester bonds in long-chain acylglycerol (C ≥ 10) (Jaeger and Reetz, 1998) resulting in the formation of an alcohol and a carboxylic acid (de Pascale et al., 2008). Most of microbial lipase showed high activity between 30°C and 50°C, while alternatively cold active lipase showed high specific activity at lower temperatures. This caused them to become a preferable choice to suit diverse industrial processes (Mayordomo et al., 2000). Due to their interesting property of high activity at low temperatures, production of frail compounds such as synthesis of chiral intermediates would make cold-active lipase as their prime choice. Structural modification in cold-active lipase by an increased flexibility of the polypeptide chain due to higher proportion of amino acids (de Pascale et al., 2012) ease substrate accommodation at low temperatures (Joseph et al., 2008). Cold-active lipase is also reported to work under low water condition due to its high flexibility. In the industry, reverse hydrolysis of substrates in low water conditions are preferable because they are able to lower down water activity besides having improved yields (Tutino et al., 2009).

Besides the need of finding novel cold-active lipase that possess industrial applications, based on protein database from (http://www.ncbi.nlm.nih.gov/protein), least numbers of cold-active lipase structures have been solved. Structure-function and catalytic activity studies of cold-active lipase will be able to help the scientific community in understanding cold adaptation. This will also lead to steps in designing and engineering lipase for specific applications in the future. Therefore, in this study, we attempt to search and identify the lipase producing ability of bacterial isolates from Arctic that are capable of producing cold-active lipase enzyme.

**Experimental**

**Materials and Methods**

**Study area and sample collection.** The field survey and sampling for this study took place during the 2011 boreal summer (August 2011) on the northern coast of Hornsund, Wedel Jarlsberg Land, West Spitsbergen in the vicinity of the Polish Polar Station "Hornsund" (77°00'04"N, 15°33'37"E). Climate of this area is strongly affected by oceanic influence. However, climatic conditions are very variable and characterized by low temperature, low precipitation and strong foehn winds. The average annual air temperature is – 4.4°C, with average monthly temperatures ranging between – 11.3°C in January and + 4.4°C in July, and average annual precipitation reaches only ca. 300–400 mm (Marsz and Styszyńska 2007).

A total of twelve sampling locations representing a range of habitats with a diversity of soil physical and chemical characteristics were sampled (data not shown). In this preliminary study, only three sample sites described in Table I were analyzed for this work. Isolates were named according to sites where they were sampled; isolate from soil sample collected at the site A were named ARA(I), isolates from sediment sample collected at the site A were named as ARA(II), and isolates from soil samples collected at the site B and C were named ARB and ARC respectively.

At each sampling location, 10 g of soil or sediment was obtained covering the surface to 10 cm depth using a sterile spatula. As for site C, soil from the bottom of the pond was sampled. The samples were collected with using a sterile spatula and placed into sealed sterile Falcon tubes. Then the samples were refrigerated at 4°C, and subsequently transported at this temperature, taking 6 days in transit to the National Antarctic Research Center, Kuala Lumpur, Malaysia, where they were stored at –20°C until further analysis (Ali et al., 2013).

<table>
<thead>
<tr>
<th>Sites</th>
<th>Designation</th>
<th>Location</th>
<th>Habitat</th>
<th>Air temperature (°C)</th>
<th>pH</th>
<th>Altitude m asl</th>
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</thead>
<tbody>
<tr>
<td>Site A (soil)</td>
<td>ARA(I)</td>
<td>Fuglebergsletta, neighbourhood of the Hornsund Station</td>
<td>Vertebrate-influenced pond</td>
<td>4.55</td>
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<tr>
<td>Site B (soil)</td>
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<td>Skjerstranda at the foothill Dried runnel of Trulsenfjellet</td>
<td></td>
<td>7.30</td>
<td>9.55</td>
<td>–</td>
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<tr>
<td>Site C (soil)</td>
<td>ARC</td>
<td>Ralstranda (southern part), at the foot of Rotjesfjellet, near Revelva river</td>
<td>Pond</td>
<td>5.85</td>
<td>8.50</td>
<td>5.24</td>
</tr>
<tr>
<td></td>
<td></td>
<td>77°00'24.12&quot; N 15°20'36.54&quot; E</td>
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</tbody>
</table>

Table I
Details of sampling locations in the Hornsund area where soil and sediment samples were collected (after Ali et al., 2013).
**Isolation of culturable bacterial strains.** An amount of 1 g of soil and sediment were inoculated into 100 ml of nutrient broth medium and grown at 15°C, 150 rpm for 15 days (Leonov, 2010). After the incubation period, the samples were serially diluted \(10^{-4}\) in sterile cold distilled water and isolated on nutrient agar plates. Single colonies obtained with obviously different macroscopic characteristics were subcultured on fresh nutrient agar plates three times to produce pure colony isolates.

**Temperature dependence study.** In order to differentiate an obligate psychrophilic and psychrotrophic strain growth, isolates obtained that were in pure colony forms were screened by growing on nutrient agar plates and incubated at three different temperatures which were 4°C (in the refrigerator), 28°C ± 2°C (at room temperature) and 37°C (in the incubator). Room temperature fluctuates at ± 2°C during day and night time.

**Plate screening for lipase.** Confirmation of lipase activity via plate screening was done by using the standard Rhodamine B fluorescent assay. Isolates were grown on Rhodamine B agar (1% v/v olive oil and 10 ml of Rhodamine B stock 0.01% w/v). Lipase production on Rhodamine B medium was monitored by orange fluorescence as seen with UV light at 350 nm. Subsequently, lipase activities on lipase positive isolates were also screened using olive oil (1% v/v) agar, palm oil (0.5% v/v) agar, tributyrin (1% v/v) agar and triolein (1% v/v agar). Halo zones seen were considered as positive for lipase production. Tween 20 (0.1% v/v) was added as inducers to all plates except the ones that used tributyrin as substrate. This is due to the fact that initial plate screening without any inducers’ addition failed to detect halo zones in all substrates of triolein, olive oil and palm oil.

**Standard curve of free fatty acids.** A series of oleic acid mixture with isoctane ranging from 0–1000 µmol was prepared in test tubes. The standard curve of free oleic acids vs. absorbance at 715 nm was determined according to Kwon and Rhee (1986). Standard curve of oleic acid was constructed to determine the lipase activity of each isolate during lipase assay.

**Lipase assay.** Fatty acids released by six lipase positive isolates as confirmed by plate screening were measured by colorimetric method according to Eltaweel et al. (2005) with modification. Cultures that had been grown for 5 days at 15°C with olive oil (1% v/v) or palm oil (0.5% v/v) as the substrate were centrifuged at 4,000 rpm to separate the cells and the extracellular enzymes. An amount of 1 ml of crude extracellular enzymes from the supernatant was mixed with 1.48 ml of 100 mM phosphate buffer pH 7, 0.02 ml of 20 mM CaCl₂, 2H₂O and 2.5 ml of olive oil emulsion in 1% poly(vinyl alcohol)(1:3 v/v). The mixture was incubated for 30 minutes at 15°C with the agitation rate of 180rpm. An amount of 1 ml of 6 N HCl and 5 ml isoctane was added to terminate the reaction. The mixture was hand shaken and allowed to separate for 10 minutes. Finally, 4 ml of the upper isoctane layer which contained liberated fatty acids were transferred to a fresh tube containing 0.2 ml of cupric acetate-pyridine reagent and further mixed by vortexing vigorously until foamed. After half an hour at room temperature, absorbance at 715 nm was determined on the isoctane that contained dissolved free fatty acids. One unit of lipase activity was equivalent to 1 µmole of fatty acid released per ml/min at 15°C.

**Morphology and molecular identification.** Morphology of the six lipase positive isolates was identified through Gram staining and molecular identification by using 16S rRNA analysis. DNA from each isolate was extracted using modified CTAB method (Sambrook and Russell, 2001). The genomic DNA of each isolate was subjected to amplification of the 16S rRNA gene using 27F and 1492R primer pairs. Sequences obtained were aligned using ClustalW (MEGA 5.10) and subjected to BLAST analysis for species identification.

**Results**

**Isolation of culturable bacteria.** A total of 63 single colony isolates were obtained from the three sample sites (Table II), comprising 21 soil isolates from site A (named as ARA(I) isolates), 8 sediment isolates from site A (named as ARA(II) isolates), 27 soil isolates from site B (named as ARB isolates) and finally 7 soil isolates from site C (named as ARC isolates).

**Temperature dependence study.** All isolates were subjected to temperature dependence study to differentiate whether they are obligate psychrophiles or psychrotrophs. Based on growth temperatures used which was 4°C, 28°C ± 2°C and 37°C, each isolate was grouped to three different groups. Isolates that only grew at 4°C were most probably obligate psychrophiles that cannot tolerate growth temperatures higher than 20°C. Isolates capable of growth at both 4°C and 28°C ± 2°C can be grouped as facultative psychrophiles or psychrotroph that tolerated growing temperatures up to 30°C. However, those that also grew at 37°C tolerated such a huge range of temperatures were not included in this study as the aim was to isolate bacteria that adapt to low temperatures. The isolates designation, growing ability at three different incubation temperatures and colony color were given in Table II.

From Site A, three soil isolates, ARA(I) 6, 15 and 19 were capable of growing at 37°C (in addition to 4°C and 28°C ± 2°C). These isolates were removed from further analyses. This left only 18 soil isolates for further lipase screening process. For some of the isolates, the growth medium turned brown/black during the isolates incubation. However, this characteristic was only recorded...
The secretion of the brownish pigment into the liquid and solid medium was analyzed through UV-Vis Spectrophotometer (UV-1800 Shimadzu) within the wavelength range of 300–800 nm with acetone as the blank. The maximum absorbance of light was measured at wavelength 320 nm, which is the wavelength absorbance for UV-B region. This high absorbance in UV-B region as well as the dark color of pigment produced (brown) was similar with the characteristics property of melanin pigment (Sajjan et al., 2010; Dastager et al., 2009). In case of sediment isolates from Site A, three pigmented sediment isolates, namely ARA(II) 1, ARA(II) 5 and ARA(II) 6, were capable of good growth at 37°C (see Table II). They were not included in the lipase screening process as well leaving only five sediment isolates for further analyses.

In site B, three isolates (ARB 16, 17 and 27) were eliminated because of their excellent growth at 37°C (see Table II). All three isolates were orange pigmented. Isolate ARB 4 failed to grow after subsequent subculturing. Therefore only 23 isolates were subjected to subsequent screening steps. All isolates from site C were subjected for further lipase screening studies.

**Plate screening for lipase.** All 53 isolates selected through temperature dependence study underwent plate screening for lipase on olive oil agar plates that used Rhodamine B as the fluorescent dye. Only six iso-

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### Table II

<table>
<thead>
<tr>
<th>Sampling site and substratum</th>
<th>Isolate</th>
<th>Incubation temperature</th>
</tr>
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<tbody>
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<td></td>
</tr>
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### Table II continued

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<td>ARB 25</td>
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<table>
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| Site C (soil)                 | ARC 1   |
|                               | ARC 2   |
|                               | ARC 3   |
|                               | ARC 4   |
| Site C (soil)                 | ARC 5   |
|                               | ARC 6   |
| Site C (soil)                 | ARC 7   |

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<td>Site C (soil)</td>
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/ Presence of growth – Absence of growth
* Blackening of the agar medium

in soil isolates from ARA (I), but not in the sediment samples of ARA (II). The secretion of the brownish pigment into the liquid and solid medium was analyzed through UV-Vis Spectrophotometer (UV-1800 Shimazdu) within the wavelength range of 300–800 nm with acetone as the blank. The maximum absorbance of light was measured at wavelength 320 nm, which is the wavelength absorbance for UV-B region. This high absorbance in UV-B region as well as the dark color of pigment produced (brown) was similar with the characteristics property of melanin pigment (Sajjan et al., 2010; Dastager et al., 2009). In case of sediment isolates from Site A, three pigmented sediment isolates, namely ARA(II) 1, ARA(II) 5 and ARA(II) 6, were capable of good growth at 37°C (see Table II). They were not included in the lipase screening process as well leaving only five sediment isolates for further analyses.

In site B, three isolates (ARB 16, 17 and 27) were eliminated because of their excellent growth at 37°C (see Table II). All three isolates were orange pigmented. Isolate ARB 4 failed to grow after subsequent subculturing. Therefore only 23 isolates were subjected to subsequent screening steps. All isolates from site C were subjected for further lipase screening studies.

**Plate screening for lipase.** All 53 isolates selected through temperature dependence study underwent plate screening for lipase on olive oil agar plates that used Rhodamine B as the fluorescent dye. Only six iso-
In the present study, all of obtained isolates were psychrophils. According to Markúsldóttir et al. (2013), psychrophils can support growth at 0°C, but have an optimal growth between 20°C to 30°C. In the present study, most of the isolates grew faster at incubation temperature 28°C ± 2°C. However, the colonies that grew at 4°C looked healthier. Isolates that are capable of withstanding a wider range of growth temperature are found in environments that experience high seasonal and/or diurnal temperature fluctuations, e.g. the sub-Arctic regions (Markúsldóttir et al., 2013). On the contrary, there are also cases of isolates from permanently cold environments that have high proportion of psychrophils. For instance, a study on the diversity of culturable bacteria in Arctic sea ice from Spitzbergen fjords revealed that most of the isolates were psychrotrophic rather than obligate psychrophiles (Groudieva et al., 2004). Sampling of the present study was conducted in summer; therefore the thawed layer of soil/sediment would select fast growing organisms capable of exploiting the moderate temperatures and higher nutrient availability (de Pascale et al., 2012).

**Plate screening for lipase.** Tributyrin was a common substrate to confirm lipase activity in most studies. However, in the present study, other longer chain fatty acids substrates were incorporated for plate screening besides the initial step that used fluorescent dye Rhodamine B to confirm lipase activities. This is due to the fact that shorter chain tributyrin can also be a target of the esterase enzyme. Lipase that possesses a broader substrate range will not only hydrolyze tributyrin, but other longer chain fatty acid substrates such as triolein, palm oil and olive oil. Unlike esterases, lipases are known to attack emulsified form of insoluble long-chain fatty acids substrate (Fojan et al., 2000). This characteristic is a consequence of the ‘interfacial activation’ phenomenon, where emulsions form cause enhancement of lipase activity (Tutino et al., 2009). Henne et al. (2000) also reported that tributyrin positive clones were unable to hydrolyze p-nitrophenyl esters during lipase assay. This is a common attribute of the esterase enzyme.

Initially, all isolates screened with olive oil, palm oil agar and triolein agar showed negative results. Therefore, there is a need for inducers presence in the medium. Tween-80 is a polyoxyethylene esters were found to induce lipase production effectively in Candida rugosa, but Tween-80 themselves do not act as carbon sources (Zhang et al., 2003). Therefore Tween 20 was added as inducers in plate screening with olive oil and palm oil carbon sources in subsequent experiments and this resulted in appearance of halo zones.

**Lipase assay.** Lipase activity is affected by the carbon length of the fatty acid and the number and position of the double bonds (Long, 2009). Olive oil with the main component of unsaturated oleic acid possess double bonds that can be broken and used easier than palm oil with higher content of saturated palmitic acid that might be harder to break. However, ARB1 and

### Table III

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Tributyrin agar (1% v/v)</th>
<th>Triolein agar (1% v/v) + Tween 20 (0.1% v/v)</th>
<th>Olive oil agar (1% v/v) + Tween 20 (0.1% v/v)</th>
<th>Olive oil agar (1% v/v) + Tween 20 (0.1% v/v)</th>
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<tbody>
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<td>ARA(I) 5</td>
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+ Presence of halo zones – Absence of halo zones.
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<th>Isolate</th>
<th>Morphology (Gram staining)</th>
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<td><em>Janthinobacterium agaricidamnosum</em> (94%)</td>
<td>Gram-negative rods</td>
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</tr>
<tr>
<td><em>Arthrobacter sulfureus</em> (98%)</td>
<td>Gram-negative rods</td>
<td></td>
</tr>
<tr>
<td>(Accession no: gi/219846645/NR_026237.1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>ARB2</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Pseudomonas migulae</em> (98%)</td>
<td>Gram-positive rods</td>
<td></td>
</tr>
<tr>
<td>(Accession no: gi/219857339/NR_024927.1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>ARB10</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Pseudomonas lurida</em> (97%)</td>
<td>Gram-negative rods</td>
<td></td>
</tr>
<tr>
<td>(Accession no: gi/343201473/NR_042199.1)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Psychrotrophic lipase producers

ARC1, which grow better on palm oil, probably have some properties that allow easy assimilation of saturated lipids. The nature of that process is unknown and requires further investigations.

**Morphology and molecular identification.** Most of the work done on bioprospecting at Arctic habitats discovered higher numbers of Gram-negative strains as compared to Gram-positive strains. Srinivas et al. (2009) reported the isolation of 38 Gram-negative strains and 9 Gram-positive strains. The low abundance of Gram-positive was due to the fact that low temperature slowed down growth of Gram-positive isolates. The Gram-negative strains in the present study were dominated by pseudomonads. Männistö and Häggblom (2006) also hypothesized that *Pseudomonas* sp. inhibits the growth of certain Gram-negative bacteria.

The resulting isolates group of this present study as determined by 16S rRNA analysis was in agreement with most of bioprospecting work on Arctic environments, where Proteobacteria, CFB (Cytophaga-Flavobacterium-Bacteroidetes) group, low and high G+C Gram-positive genera (Srinivas et al., 2009, Reddy et al., 2009, Groudieva et al., 2004) predominates. In the present study, lipase producing isolates were dominated by Proteobacteria; particularly Gammaproteobacteria where four of the isolates were pseudomonads. Pseudomonads are well known for their metabolic versatility, simple nutrient requirements and genetic adaptability (Markúsdóttir et al., 2013). Most of the pseudomonads from this group bear closest similarities with *Pseudomonas fluorescens* species group, a common isolate found in cold habitats. Meyer et al. (2004) reported the isolation of high numbers of *Pseudomonas sensu stricto* genus in the cold alpine Colorado soil, in which *P. fluorescens* group falls into the main cluster.

In the present study, another group of betaproteobacteria, *Janthinobacterium agaridamnosum* was also found to produce lipase. Tindall et al. (2000) isolated two strains of *Janthinobacterium lividum* (clusters 1a and 1b) from a mat in Dry Valleys, Antarctica. *Janthinobacterium agaridamnosum* isolated from the present study was related to *Janthinobacterium lividum*. These are two well known species in the genus *Janthinobacterium* (Tindall 2004).

Psychrotrophic *Arthrobacter* strains were usually found in the Arctic soils, different Antarctic environments and on various glaciers. They were also abundant in subterranean cave slits (Margesin et al., 2004). Juck et al. (2000) reported high G+C Gram-positive cold-adapted microorganisms especially from the genera *Arthrobacter* dominating hydrocarbon contaminated soils; believed to play a role in hydrocarbon degradation.

Strong research interest in cold-active lipase has led to efforts in cloning and expression of respective lipase...
genes in the lab currently. Besides that, ecological signif-
nicance of the lipase enzyme that was present in these
isolates needs further investigation especially on the
soil composition of the sampling sites. This is due to the
need to discover the source and types of lipids used by
these isolates.

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arctica Research Program (MARP) and University of Malaya (UM)
(OCAR TNC(P&R) 2011 Account No. (A-55001-DA000-B21520).

Literature
and P. Convey. 2013. Studies on diversity of soil microfungi in the
Dastager S., W.-J. Li, A. Dayanand, S.-K. Tang, X.-P. Tian,
tion and analysis of pigment (melanin) production in Streptomyces.

de Pascale D., A.M. Cusano, F. Autore, E. Parrilli, G. di Prisco,
G. Marino and M.L. Tutino. 2008. The cold-active Lip1 lipase from
the Antarctic bacterium Pseudoalteromonas haloplanktis TAC125 is
a member of a new bacterial lipolytic enzyme family. Extremophiles
12: 311–323
de Pascale D., C. de Santi, J. Fu and B. Landfald. 2012. The microbial
diversity of Polar environments is a fertile ground for bioprospecting. Marine Genomics 8: 15–22
2005. An organic solvent-stable lipase from Bacillus sp. strain 42.
What distinguishes an esterase from a lipase: a novel structural
analysis and differentiation of pigments (melanin) production in
Groudeva T., M. Kambourova, H. Yusef, M. Royter, R. Grote,
What distinguishes an esterase from a lipase: a novel structural
analysis and differentiation of pigments (melanin) production in

method for determination of free fatty acids for lipase assay. JAOCS
63: 89–92.

Leonov S.I. 2010. Screening for Novel Cold-active Lipases from
Wild Type Bacteria Isolates. Innovative Romanian Food Biotechnol-
ogy 6: 12–17.

Long K. 2009. Unlocking the miracle of lipases. Research Inaugu-
ral Lecture at Auditorium Mardi Serdang on the 22 October 2009.
.my/doc/document_library/get_file?uid=7106db4-3661-4301-a994-
d88272dd56&groupid=10138

Männistö M.K. and M.M. Häggblom. 2006. Characterization of
psychrotolerant heterotrophic bacteria from Finnish Lapland. Sys-

Arthrobacter psychrophilicus sp. nov., isolated from an alpine ice
cave. International Journal of Systematic and Evolutionary Microbio-
logy, 54: 2067–2072

Markusdottir M., S. Heiðmarsson, A. Eyrósdóttir, K.P. Mag-
nússon and O. Vilhelmsen. 2013. The natural and anthropogenic
microbiota of Glæra, a sub-arctic river in northeastern Iceland. Inter-
national Biodeterioration and Biodegradation 84: 192–203.

Marz A. and A. Styszyńska. 2007. Klimat rejonu Polskiej Stacji
Polańce w Hornsundzie – stan, zmiany i przyczyny (Climate of
the Polish Polar Station in Hornsund – state, changes and rea-
sions). Wydawnictwo Akademii Morskiej w Gdyni, Gdynia: pp. 376
(in Polish)

Mayordomo I., F. Randez-Gil and J.A. Prieto. 2000. Isolation,
Purification, and Characterization of a Cold-Active Lipase from

2004. Molecular and metabolic characterization of cold-tolerant
alpine soil Pseudomonas sensu stricto. Applied Environmental
Microbiology 70: 483–489.

Reddy P.V., S.S.S.N. Rao, M.S. Pratibha, B. Sailaja, B. Kavya,
Bacterial diversity and bioprospecting for cold-active enzymes from
culturable bacteria associated with sediment from a melt water
stream of Midre Lovenbreen glacier, an Arctic glacier. Research in
Microbiology 160: 538–546.

Sajjan S., V. Yaligara and T. Karegoudar. 2010. Purification and
Physiochemical Characterization of Melanin Pigment from Kleb-
siella sp. GSK. Journal of Microbiology and Biotechnology 20(11),
1513–1520.

tory manual. 3rd edition; Cold Spring Harbor Laboratory; New York.
Srinivas, T.N.R., S.S.S.N. Rao, P.V.V. Reddy, M.S. Pratibha,
B. Sailaja, B. Kavya, K. Hara Kishore, Z. Begum, S.M. Singh and
S. Shivaji. 2009. Bacterial Diversity and Bioprospecting for Cold-
Active Lipases, Amylases and Proteases, from Culturable Bacteria of
Kongsfjorden and Ny-Ålesund, Svalbard, Arctic. Current Microbiol-
ogy 59: 537–547

Tindall B.J. 2004. Prokaryotic Diversity in the Antarctic: The Tip of
the Iceberg Microbial Ecology 47: 271–283

Tindall B.J., E. Brambilla, M. Steffen, R. Neumann, R. Pukall,
M.R. Kopperstedt and E. Stackebrandt. 2000. Cultivable micro-
bial biodiversity; gnawing at the Gordian knot. Environmental Micro-
biology 2(3): 310–318

Tutino M.L., G. Prisco, G. Marino and D. Pascale. 2009. Cold-

Yu Y., H. Li, Y. Zeng and B. Chen. 2009. Extracellular enzymes
of cold-adapted bacteria from Arctic sea ice, Canada Basin. Polar
Biology 32: 1539–1547

lipase production by Candida rugosa. Annals of Microbiology 53 (4):
499–504
Plant Growth Promotion Rhizobacteria in Onion Production

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Abstract

The aim of the research was to examine the effect of rhizospheric bacteria Azotobacter chroococcum, Pseudomonas fluorescens (strains 1 and 2) and Bacillus subtilis on the growth and yield of onion and on the microorganisms in the rhizosphere of onion. The ability of microorganisms to produce indole-acetic acid (IAA), siderophores and to solubilize tricalcium phosphate (TCP) was also assessed. The experiment was conducted in field conditions, in chernozem type of soil. Bacillus subtilis was the best producer of IAA, whereas Pseudomonas fluorescens strains were better at producing siderophores and solubilizing phosphates. The longest seedling was observed with the application of Azotobacter chroococcum. The height of the plants sixty days after sowing was greater in all the inoculated variants than in the control. The highest onion yield was observed in Bacillus subtilis and Azotobacter chroococcum variants. The total number of bacteria and the number of Azotobacter chroococcum were larger in all the inoculated variants then in the control. The number of fungi decreased in most of the inoculated variants, whereas the number of actinomycetes decreased or remained the same.

Keywords: growth and yield of onion, PGP rhizobacteria, rhizospheric microorganisms

Introduction

Plant growth promoting rhizobacteria (PGPR) affect plant growth directly or indirectly by producing growth substances such as indole-acetic acid, gibberelric acid and cytokinins (Verma et al., 2010; Garcia de Salamone et al., 2001), fixing dinitrogen from the atmosphere and providing the plant with this element (Boddey and Dobereiner, 1995) and by being antagonistic towards phytopathogenic microorganisms (Velivelli et al., 2012). In recent decades, different PGPR have been studied, including nitrogen-fixing bacteria from Azotobacter genus and bacteria which produce growth substances and act as antagonists, such as Bacillus and Pseudomonas (McSpaden-Gardener, 2004; Benizri et al., 1998). The interaction between rhizobacteria and plants is not always stable in nature, thus positive results obtained in controlled conditions cannot always be replicated in field conditions (Jarak et al., 2012). The effect of PGPR varies as a result of environmental factors, which may affect both the growth of bacteria and the plant. The effect of the introduced bacteria also depends on plant physiology and agronomic conditions of cultivation. In order to achieve an optimum interaction between rhizobacteria and the plant root, it is necessary to examine the way in which rhizobacteria affect the plant and microorganisms in soil and whether this influence changes due to environmental factors, including the presence of other microorganisms, as well (Stamenov et al., 2012a).

Onion (Allium cepa) is an important vegetable plant. Due to its high adaptability, there are numerous populations and varieties of onion grown under various environmental conditions. Onion contains a large amount of carbohydrates and a small amount of proteins and fats. The biological value of onion lies in its mineral substances and vitamins. Apart from a significant amount of mineral salts, especially potassium and sulphur salts, and different oligoelements, onion abounds in vitamins (B1, B2, C, E, K), carotene (provitamin A), glycosides, etheric oils, plant hormones similar to insulin, as well as bacteriostatics (Slimestad et al., 2007). The root system of onion has low absorbing and penetrating abilities, therefore it requires an ample amount of easily accessible nutrients in the root zone. The amount of easily accessible nutrients which is necessary for optimum yield is 60–140 kg N, 60–120 kg P₂O₅ and 60–180 kg K₂O per hectare (Kumar, 2001). Recently,
plant growth promoting rhizobacteria (PGPR) have come into focus as bacteria which can provide a part of necessary nutrients.

The aim of the study was to examine the effect of PGPR _Bacillus subtilis_, _Pseudomonas fluorescens_ and _Azotobacter chroococcum_ on germination, growth and yield of onion, as well as on the microbiological activity in the rhizosphere during the vegetation period of onion.

**Experimental**

**Material and Methods**

**Bacterial strains.** The bacteria used in the study included _Azotobacter chroococcum_, _Pseudomonas fluorescens_ strain 1, _Pseudomonas fluorescens_ strain 2 and _Bacillus subtilis_ (from Department of Microbiology, Faculty of Agriculture, Novi Sad, Serbia). The microorganisms were propagated in appropriate nutrient media (Handbook of Microbiological Culture Media, 2000): _Azotobacter chroococcum_ in nitrogen-free medium with mannitol, _Pseudomonas fluorescens_ in King B medium (Himedia, India), and _Bacillus subtilis_ in nutrient agar (NA) (Torlak, Serbia).

**Indole-acetic acid (IAA) production.** IAA production was examined using Gordon and Weber method (Gordon and Weber, 1951). Using Salkowski reagent (1 ml 0.5 M FeCl$_3$ in 50 ml 35% HClO$_4$) the production of IAA in a medium containing 0, 200 and 500 µg/ml of L-tryptophane was determined. The volumes of 100 µl of 24 h bacterial culture (standardized to OD$_{600}$ of 0.625), were introduced into 100 ml of liquid media: _Pseudomonas_ into King B medium, _Bacillus_ into NA medium and _Azotobacter_ into nitrogen-free medium with mannitol. After 24 h and 48 h, incubation at 28°C, 5 ml of the suspension was centrifuged at 1,957 x g for 15 minutes. An amount of 2 ml of Salkowski reagent was added into 1 ml of supernatant. Twenty-five minutes later, the intensity of the development of pink colour was measured at 530 nm.

**Phosphate solubilization.** The ability of isolates to solubilize tricalcium phosphate (TCP) (CaCO$_3$(PO$_4$)$_2$) was investigated in a Pikovskaya medium (amount g l$^{-1}$: KCl 0.2 g, MgSO$_4$ x 7H$_2$O 0.1 g, glucose 10 g, yeast extract 0.5 g, FeSO$_4$ x 7H$_2$O 0.002 g, MnSO$_4$ x H$_2$O 0.002 g, (NH$_4$)$_2$ SO$_4$ 0.5 g, Ca$_2$(PO$_4$)$_2$ 5 g). An amount of 1 ml bacterial culture, respectively (standardized to OD$_{600}$ of 0.625) was introduced into the cooled medium, poured into a Petri dish and carefully mixed. After five days of incubation at the temperature of 28°C, transparent zones around the colonies were measured.

**Siderophore production.** The ability of siderophore production was determined by the method of Milagres _et al._ (1999), using chrome azurol agar (CAS agar). Appropriate nutrient media for _Bacillus_, _Pseudomonas_ and _Azotobacter_ were poured into Petri dishes. After they had solidified, the media were cut into halves and one half was removed. CAS agar was poured into the empty halves of the Petri dishes. The bacteria were introduced into the halves with the nutrient medium. The incubation lasted five days at the temperature of 28°C. In those strains which produce siderophores, the blue-green colour of CAS agar turned into orange along the demarcation line between the two media.

**Field experiment.** The experiment was conducted in Backi Brestovac (Vojvodina, Serbia), in carbonate chernozem soil. The soil was characterized by the following properties: pH in H$_2$O: 8.06, pH in KCl: 7.19, % CaCO$_3$: 4.62, % humus: 2.63, % N: 0.13, mg P$_2$O$_5$/100 g soil: 22.27, mg K$_2$O/100 g soil: 18.12. The experimental design was a randomized, complete block with four replications. The size of the experimental plots was 10 m$^2$ (10 m long, 1 m wide). The sowing was performed on 12 March, 2012. The spacing in a row was 5 cm and 25 cm between the rows.

**Bacterial treatments.** The onion seed (Damascus f1 hybrid, Holland) was inoculated with four bacterial strains: 1. _Azotobacter chroococcum_; 2. _Bacillus subtilis_; 3. _Pseudomonas fluorescens_, strain 1; 4. _Pseudomonas fluorescens_, strain 2; and with a mixture of strains _Azotobacter chroococcum_ + _Bacillus subtilis_ + _Pseudomonas fluorescens_, strain 1+ _Pseudomonas fluorescens_, strain 2 (ratio 1:1:1:1). The control was not inoculated. An amount of 50 ml of the inoculum having the density of 10$^4$/ml was introduced into 200 g of sterile peat. The inoculated peat was applied into rows, directly to the onion seed. Standard agrotechnical practices were applied during the vegetation period.

**The effect of inoculation on onion growth.** The germ growth was observed five and ten days after the inoculation. The onion seeds were washed with sterile tap water and placed on plastic trays with a moist filter paper. The trays were covered and placed to the incubator at 28°C. After 48 hours the shoots of equal length were selected. The onion roots were dipped into a bacterial suspension (10$^4$ CFU/ml) for 1.5 hours. In the control the roots were moistened in sterile water. Then the onion seedlings were placed on the tray with a moist filter paper, covered and incubated at 28°C. After five and ten days the length of the roots and the shoots was measured.

The plant height and the dry mass were measured three months after the sowing (the phase of 5–6 leaves). The bulb size and the bulb weight were measured three months after the sowing and at the end of the vegetation period (early August). The onion yield (t/ha) was determined at the end of the vegetation period.
The effect of inoculation on the microbial population in the rhizosphere of onion. The rhizospheric soil was sampled for the purpose of microbial analysis 30, 90 and 150 days after sowing. The number of microorganisms was determined by the method of agar plates in the appropriate nutrient medium: The total microbial count was performed in soil agar, (dilution 10−6), the fungi in potato-dextrose agar (Hy media) (dilution 10−4), the number of actinomycetes in Krasilnikov agar (Hy media) (dilution 10−5), the total number of bacteria in nutrient agar (NA) (Törlak, Belgrade) (dilution 10−4) and the number of azotobacter in Fiodorov medium (Hy media) (dilution 10−3). All microbial analyses were performed in three replications and the average number for all three samplings was calculated per 1.0 g of absolutely dry soil.

Data analysis. Statistical analysis was performed by using the statistical software STATISTICA, version 12.0 (Hamburg, Germany). The significance of each treatment was established by one way ANOVA and the means were separated by Fisher’s test (P ≤ 0.05).

Results and Discussion

Indole-acetic acid (IAA) production. Indole-acetic acid (IAA) is the main auxin in plants, controlling many important physiological processes including cell enlargement and division, tissue differentiation, and responses to light and gravity. Diverse bacterial species (Bacillus spp, Streptomyces sp., Rhizobium sp., Azotobacter, Pseudomonas) possess the ability to produce IAA (Ahmad et al., 2005). According to Loper and Schorth (1986), 80% of bacteria isolated from the rhizosphere are capable of producing IAA. In our research, all the strains were producing IAA. The best production was observed after 48 hours of incubation in the presence of 500 µg/ml of L-tryptophan (Table I). The best producer of IAA was Bacillus subtilis.

Phosphate solubilization and siderophore production. Phosphorus is found in soil in its organic and inorganic compounds. PGPR play a role in transforming inaccessible compounds (both organic and inorganic) into forms accessible to plants. Bacterial strains belonging to the genera Pseudomonas, Bacillus, Rhizobium, Azotobacter, Achromobacter, Agrobacterium, Micrococcus, Aerobacter, Flavobacterium and Erwinia are capable of solubilizing insoluble phosphate compounds such as tricalcium phosphate and dicalcium phosphate (Rodriguez and Fraga, 1999). Our research has shown that both Pseudomonas fluorescens strains are better TCP solubilizers than Bacillus and Azotobacter (Table II). Ravindra Naik et al., (2008) tested 443 strains of Pseudomonas. Their research showed that 80 strains (18%) produced phosphate solubilization on Pikovskaya’s agar medium by inducing clear zones. Fluorescent pseudomonad strains have also been reported as phosphate solubilizers due to the excretion of organic acids by many other researchers (Bano and Musarrat, 2004; Cattelan et al., 1999; Pandey and Palani, 1998).

Siderophores are high-affinity Fe3+ chelating compounds. In cases when accessible iron is lacking, plants and microorganisms produce siderophores which take Fe3+ out of its compounds, bind it and form a Fe3+ siderophore complex. This complex is transported to the surface of a bacterial cell or root cells, transported into the cell and reduced to Fe2+. Plants are capable of binding the bacterial Fe2+ siderophore complex, thus PGPR help in providing the plant with iron (Kalinowski et al., 2000). It has been proved that a large number of rhizospheric microorganisms, including Bacillus subtilis, Pseudomonas sp. and Azotobacter sp., produce siderophores (Jankiewicz, 2006, Jarak et al., 2012). In this research, Pseudomonas fluorescens strains were better siderophore producers (Table II). This is in accordance to investigation of Djurić et al. (2011). In their work, three siderophore-producing isolates of Pseudomonas fluorescens were detected. Parani and Saha (2012) also proved the existence of three siderophore producing Pseudomonas strains.

Onion growth. As PGPR produce plant hormones, the use of these bacteria usually enhances germination and early plant growth. In this research, the seed inoculation affected onion germination and growth

<table>
<thead>
<tr>
<th>Microorganisms</th>
<th>after 24 h L-tryptophan µg/ml</th>
<th>after 48 h L-tryptophan µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>200</td>
</tr>
<tr>
<td>Pseudomonas fluorescens 1</td>
<td>2.42</td>
<td>5.39</td>
</tr>
<tr>
<td>Pseudomonas fluorescens 2</td>
<td>1.93</td>
<td>3.18</td>
</tr>
<tr>
<td>Bacillus subtilis</td>
<td>12.07</td>
<td>19.75</td>
</tr>
<tr>
<td>Azotobacter chroococcum</td>
<td>3.73</td>
<td>6.23</td>
</tr>
</tbody>
</table>

The datas are mean values of three repetitions.
Five days after the inoculation, the seedling was longer in all the inoculated variants than in the control, whereas ten days after the inoculation, the stimulating effect was visible in the variants with Azotobacter chroococcum and Pseudomonas fluorescens strains. Sixty days after the inoculation the length of the part above the ground was greater in all the inoculated variants than in the control. The fresh mass of the part above the ground was smaller in the variants inoculated with Pseudomonas fluorescens strains, whereas the fresh mass of the bulb was greater in all the inoculated variants (apart from the variant with the mixture of strains). However, the increase was not statistically significant. This could be explained by the high variability of the data within treatments. The effect of the treatments was not strong enough to produce a statistically significant result.

Similar results were shown by Jarak et al. (2006) who reported a better germ development and early growth of alfalfa and red clover with the use of azotobacter, rhizobia and actinomycetes. Similarly, Stamenov et al. (2012b) reported that PGPR enhance the growth of English ryegrass. Joo et al. (2005) inoculated the pepper seed with Penibacillus polymyxa and Bacillus subtilis. In all the inoculated variants, the length and the mass of the above ground part and root significantly increased.

Onion yield. At the end of the vegetation period, onion bulbs were taken out and their size and yield were measured (Table V). The bulb diameter was larger in all the inoculated variants but the increase was of no statistical significance. The bulb weight and the yield significantly increased in the variants with Azotobacter chroococcum, Bacillus subtilis and the mixture of the inoculants. Both Azotobacter chroococcum and Bacillus subtilis take part in other important processes apart from promoting plant growth. Azotobacter can fix 60–80 kg N/ha and thus partly provide the plant with this element (Yanni and El-Fattah, 1999, Kennedy et al., 2004), whereas Bacillus subtilis is a bioagent against phytopathogenic fungi (Kloepper et al., 2004). These properties certainly promote plant growth and increase onion yield. The use of azotobacter results in an increased yield of other plant species, too. Kumar et al. (2001), and Hajnal Jafari et al. (2012) reported that the use of azotobacter led to an increased yield of wheat and maize. The research conducted in field experiments by Mrkovacki et al. (2012) showed that inoculation of sugar beet with Azotobacter chroococcum resulted in an
increased root yield and amount of extractable sucrose from the sugar beet. Nieto and Frankenberger (1991) investigated the effect of *Azotobacter chroococcum* on the morphology and growth of maize in vitro, in a greenhouse and in the field, and concluded that the plant growth was enhanced thanks to hormone production by azotobacter.

**Microbial population in onion rhizosphere.** The microbial diversity in the rhizosphere depends on root exudates, soil properties, agrotechnical measures and ecological factors. In this research, the number of the investigated groups of microorganisms was large and typical of fertile soils such as chernozem (Table VI).

Plants exude various organic and inorganic substances through their roots into the rhizosphere. The larger the number of monosaccharides and easily disintegrable organic acids in the rhizosphere, the larger the number of rhizospheric microorganisms. Onion roots exude aminoacids, sugars and organic acids which influence positively the rhizosphere (Tawaraya *et al*., 1995) creating favorable conditions for different microorganisms. Introducing microbiological fertilizers into soil can change the number and species of the indigenous microbial population. In this research, the total number of microorganisms (apart from treatment indigenous microbial population. In this research, the number of the investigated groups of microorganisms was large and typical of fertile soils such as chernozem (Table VI).

Table VI

<table>
<thead>
<tr>
<th>Treatment</th>
<th>TN 10⁶g⁻¹</th>
<th>TB 10⁶g⁻¹</th>
<th>AC 10⁶g⁻¹</th>
<th>F 10⁶g⁻¹</th>
<th>AZ 10⁶g⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Azotobacter chroococcum</em></td>
<td>329.00</td>
<td>249.90</td>
<td>11.94</td>
<td>3.05</td>
<td>168.01</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>1069.90</td>
<td>1227.28</td>
<td>26.05</td>
<td>24.72</td>
<td>153.05</td>
</tr>
<tr>
<td><em>Pseudomonas fluorescens</em> 1</td>
<td>63.94</td>
<td>1039.75</td>
<td>11.51</td>
<td>4.80</td>
<td>106.06</td>
</tr>
<tr>
<td><em>Pseudomonas fluorescens</em> 2</td>
<td>439.82</td>
<td>995.24</td>
<td>29.92</td>
<td>10.33</td>
<td>115.34</td>
</tr>
<tr>
<td>Mixture</td>
<td>663.66</td>
<td>1029.96</td>
<td>24.99</td>
<td>3.61</td>
<td>109.50</td>
</tr>
<tr>
<td>Control</td>
<td>165.40</td>
<td>95.57</td>
<td>23.79</td>
<td>14.34</td>
<td>70.20</td>
</tr>
<tr>
<td>LSD 0.05</td>
<td>196.55</td>
<td>215.80</td>
<td>6.08</td>
<td>3.50</td>
<td>29.42</td>
</tr>
</tbody>
</table>

The datas are mean values of three repetitions.

TN – total number of microorganisms; TB – total number of bacteria; AC– number of actinomycetes; F– number of fungi; AZ– number of azotobacter

used as biocontrol agents because they protect plant roots from phytopathogenic fungi such as *Fusarium* and *Pythium*. The introduction of these microorganisms into the rhizosphere leads to activation of useful microbiological processes, increase in the number of bacteria and decrease in the number of fungi. These PGPR species are already being used in the production of wheat, maize, sugar beet, rice, pepper, tomato, cucumber etc. The obtained results have shown that PGPR can also be used in onion production.

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**Literature**


The Geographical Range of Tick-Borne Diseases (TBD), Identification of New Pathogens, as Well as the Increase of TBD Incidence Makes TBD a Serious Public Health Problem. Besides Lyme Borreliosis and Tick-Borne Encephalitis, Which Are Monitored in Poland, Other TBD Caused by Babesia spp., Rickettsia spp., and Anaplasma phagocytophilum, Are of Increasing Public Health Interest.

Anaplasma phagocytophilum, a Small Gram-Negative Obligate Intracellular Alfaproteobacteria, Is a Tick-Borne Rickettsial Bacterium That Replicates in Mammalian Granulocytes but Also in the Salivary Gland and Midgut Cells of Ticks (Rikihisa, 2011). It Causes Human Granulocytic Anaplasmosis (HGA), Previously Known as Human Granulocytic Ehrlichiosis (HGE), But Also the Disease in Horses, Dogs, Sheep, and Cats (Rar and Golovljova, 2011; Severo et al., 2012). HGA Is an Emerging Disease with Varying Symptoms, from Asymptomatic Seroconversion to Non-Specific Symptoms Like Fever, Malaise, Myalgia and Headache, Up to Fatal Disease (Dumler et al., 2005; Zwoliński et al., 2007; Rar and Golovljova 2011). The Majority of Confirmed HGA Cases, Including the First Case of HGA Recorded in the Early 1990s (Rikihisa, 2011), Have Been Reported from the USA. In Europe, the Prevalence of HGA Infection Is Significantly Lower, Although Human Cases Have Been Accounted for the Majority of European Countries, Including Poland (Tylewska-Wierzbanowska et al., 2001; Grzeszczuk et al., 2009; Rikihisa, 2011).

Anaplasma phagocytophilum DNA Has Been Detected in Several Species of Hard Ticks (Ixodes scapularis, I. pacificus, I. spinipalpis, I. ricinus, I. persulcatus, I. trianguliceps, I. ventlalloi, I. oculus, Dermacentor silvarum) in the United States, Europe, and Asia (Rar and Golovljova 2011; Rikihisa, 2011). In Poland, but Also All Over Europe, I. ricinus, the Most Prevalent Tick Species Responsible for the Majority of Tick Bites in Humans, Is the Main Vector of A. phagocytophilum. The Aim of the Study Was to Estimate the Infection Level of I. ricinus with A. phagocytophilum in Selected Districts, Not Previously Surveyed for the Presence of This Agent. Sampling of Questing Ticks Was Performed in 12 Forested Sites, Located in Four Districts (Legnica, Milicz, Lubań, and Olawa) in SW Poland. Altogether, 792 Ticks (151 Females, 101 Males, and 540 Nymphs) Representing I. ricinus Were Checked for the Presence of A. phagocytophilum. The Average Infection Level Was 4.3%, With Higher Rate Reported for Adult Ticks. The Highest Percentage of Infected Adult Ticks Was Observed in Milicz (17.4%) and the Lowest in Olawa (6.8%). The Abundance of Questing I. ricinus in All Examined Sites as Well as the Infection with A. phagocytophilum Indicate for the First Time the Risk for HGA Transmission in SW Poland.

Key words: Anaplasma phagocytophilum, Ixodes ricinus, Poland

Introduction

The geographical range of tick-borne diseases (TBD), identification of new pathogens, as well as the increase of TBD incidence makes TBD a serious public health problem. Besides Lyme borreliosis and tick-borne encephalitis, other pathogens such as Babesia spp., Rickettsia spp., and Anaplasma phagocytophilum, are of increasing public health interest. In Poland, as in other European countries, Ixodes ricinus, the most prevalent tick species responsible for the majority of tick bites in humans, is the main vector of A. phagocytophilum. The aim of the study was to estimate the infection level of I. ricinus with A. phagocytophilum in selected districts, not previously surveyed for the presence of this agent. Sampling of questing ticks was performed in 12 forested sites, located in four districts (Legnica, Milicz, Lubań, and Olawa) in SW Poland. Altogether, 792 ticks (151 females, 101 males, and 540 nymphs) representing I. ricinus were checked for the presence of A. phagocytophilum. The average infection level was 4.3%, with higher rate reported for adult ticks. The highest percentage of infected adults was observed in Milicz (17.4%) and the lowest in Olawa (6.8%). The abundance of questing I. ricinus in all examined sites as well as the infection with A. phagocytophilum indicate for the first time the risk for HGA transmission in SW Poland.

The Prevalence of Anaplasma phagocytophilum in Questing Ixodes ricinus Ticks in SW Poland

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Abstract

Ticks constitute important vectors of human and animal pathogens. Besides the Lyme borreliosis and tick-borne encephalitis, other pathogens such as Babesia spp., Rickettsia spp., and Anaplasma phagocytophilum, are of increasing public health interest. In Poland, as in other European countries, Ixodes ricinus, the most prevalent tick species responsible for the majority of tick bites in humans, is the main vector of A. phagocytophilum. The aim of the study was to estimate the infection level of I. ricinus with A. phagocytophilum in selected districts, not previously surveyed for the presence of this agent. Sampling of questing ticks was performed in 12 forested sites, located in four districts (Legnica, Milicz, Lubań, and Olawa) in SW Poland. Altogether, 792 ticks (151 females, 101 males, and 540 nymphs) representing I. ricinus were checked for the presence of A. phagocytophilum. The average infection level was 4.3%, with higher rate reported for adult ticks. The highest percentage of infected adults was observed in Milicz (17.4%) and the lowest in Olawa (6.8%). The abundance of questing I. ricinus in all examined sites as well as the infection with A. phagocytophilum indicate for the first time the risk for HGA transmission in SW Poland.

Key words: Anaplasma phagocytophilum, Ixodes ricinus, Poland

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through a blood meal, and they can maintain the bacterium through subsequent molts (transstadial passage), but not by transovarial transmission (Rikihisa, 2011). In Europe, a wide range of mammalian species is infected with *A. phagocytophilum* (Rar and Golovlyova, 2011). However, it is worth noting that the zoonosis potential and host infectivity of *A. phagocytophilum* depend on genetic variations of strain, due to the host tropism of this bacterium (Severo et al., 2012). Thus, humans are susceptible to limited strains only. The wild host reservoir for strains that infect humans (Ap-ha) is poorly known (Massung et al., 2002; Rikihisa, 2011; Michalik et al., 2012).

The aim of the study was to estimate the infection level of *I. ricinus* with *A. phagocytophilum* in selected districts of Lower Silesia, SW Poland, not hitherto surveyed for the presence of this agent.

**Experimental**

**Materials and Methods**

**Tick collection.** Sampling of questing ticks was performed in 12 forested sites, located in four districts in SW Poland: Lubań (sites: L1, L2, L3), Milicz (M1, M2, M3), Legnica (Leg1, Leg2, Leg3) and Oława (O1, O2, O3). Ticks were collected by flagging method during spring peak of tick activity, from April to June 2011, at least once a month in each site. Collected ticks were placed in plastic tubes and kept in the refrigerator. The identification of species and life instar was carried out in stereomicroscope, based on the key provided by Siuda (1993).

**Detection of *A. phagocytophilum* in *I. ricinus* ticks.** DNA for PCR was extracted from *I. ricinus* by lysis in ammonium hydroxide (Rijkpema, 1996). The isolates from nymphs and adults were used for the detection of *A. phagocytophilum*. Due to the predominance of nymphs in the population, the PCR reactions for nymphs were carried out in pools (5 specimens per pool), which increased the representation of this particular instar and reduced the costs of analysis. Thus, for the calculation of the level of infection in nymphs the minimal infection rate (MIR) was used (calculated on the assumption that in each positive pool of tested nymphs only one was infected). The procedure was carried out for 30 samples from each of the 12 monitored sites. To estimate the presence of *A. phagocytophilum* the diagnostic kit (PK24N DNA Gdańsk) was used. The detection was based on amplification of 16S rDNA by nested PCR reaction. The reaction was performed in a reaction volume of 50 μl containing 42 μl Master Mix PCR-OUT for first and PCR-IN for second amplification, 5 μl dNTPs mixture, 1 μl DNA polymerase *TaqNova*, and 2 μl of the processed tick sample or 2 μl PCR product for nested-PCR. The positive control contained *Anaplasma* DNA from a diagnostic kit, whereas the negative control contained sterile water. The amplification was performed in a BioRad T100™ Thermal Cycler. The amplification included initial denaturation for 2 min at 95°C, subjected to 40 cycles (for a second reaction 15 cycles) of 30 s denaturation at 94°C, 30 s for the annealing reaction at 55°C, 60 s extension at 72°C and final extension for 5 min at 72°C. The separation of the nested PCR products was carried out on a 1.5% agarose gel with the addition of ethidium bromide in the TBE buffer. Product of 546 bp was regarded as a positive result.

**Statistical analysis.** The results were analysed using STATISTICA v.10 software. The χ² test, Kruskal-Wallis ANOVA test and Mann-Whiteney test were applied. Probability at p < 0.05 was regarded as significant.

**Results**

In total, 2507 host-seeking ticks (148 larvae, 1314 nymphs, 478 females, and 567 males) identified as *Ixodes ricinus* were collected in four districts. Ticks were present in each of 12 sites. However, the tick density varied between districts (H = 235.2; p < 0.01) with the highest prevalence in Lubań (Table I). Altogether, 792 *I. ricinus* ticks (151 females, 101 males, and 540 nymphs) were tested for the presence of *A. phagocytophilum*. Infected ticks were found in all districts. The average infection rate was 4.3%. The infection rate in adults was higher than in nymphs (χ² = 37.093; p < 0.001). The minimum infection rate was 1.3% in nymphs and 10.7% in adults (Table II). The highest level of nymph infection was recorded in Legnica district (3.1%), while no infected nymphs were found in Lubań district. The highest rate of infected adults was found in Milicz (17.4%) and the lowest one in Oława (6.8%). However, the differences between districts were not statistically significant, both for nymphs and adults (χ² = 4.321; p = 0.229, χ² = 3.547; p = 0.314, respectively). A higher infection rate was observed in females in comparison to males (15.2% and 3.9% respectively).

**Table I**

The average density of *I. ricinus* in four districts in south-western Poland from April to June 2011.

<table>
<thead>
<tr>
<th>District</th>
<th>The average number of <em>I. ricinus</em> ticks collected by one person in 30 min</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
</tr>
<tr>
<td>Lubań</td>
<td>26.5</td>
</tr>
<tr>
<td>Milicz</td>
<td>10.2</td>
</tr>
<tr>
<td>Legnica</td>
<td>3.4</td>
</tr>
<tr>
<td>Oława</td>
<td>4.0</td>
</tr>
</tbody>
</table>
Infection level of *I. ricinus* ticks with *A. phagocytophilum* in SW Poland

**Table II**

*Anaplasma phagocytophilum* infected *I. ricinus* ticks in four districts in south-western Poland in 2011.

<table>
<thead>
<tr>
<th>District</th>
<th>nymphs*</th>
<th>females</th>
<th>males</th>
<th>females &amp; males</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>number of tested</td>
<td>positive (%)</td>
<td>number of tested</td>
<td>positive (%)</td>
</tr>
<tr>
<td>Luban</td>
<td>170</td>
<td>0</td>
<td>37</td>
<td>10.8</td>
</tr>
<tr>
<td>Milicz</td>
<td>220</td>
<td>1.8</td>
<td>23</td>
<td>26.1</td>
</tr>
<tr>
<td>Legnica</td>
<td>65</td>
<td>3.1</td>
<td>47</td>
<td>17.0</td>
</tr>
<tr>
<td>Oława</td>
<td>85</td>
<td>1.2</td>
<td>44</td>
<td>11.4</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>540</td>
<td>1.3</td>
<td>151</td>
<td>15.2</td>
</tr>
</tbody>
</table>

* nymphs were tested in pools consisting of five specimens

χ² = 8.037; p = 0.004). The highest level of infection in females was found in Milicz and in Legnica districts (26.1% and 17.0% respectively), and the lowest one (10.8%) – in Luban district. The infection level in males varied between 0 (Oława district) and 8.7% (Milicz district). There were no statistically significant differences between the density of adult ticks in districts, and the level of their infection (Z = −0.282; p = 0.778)

**Discussion**

The occurrence of questing *Ixodes ricinus* ticks is a major factor of tick-borne infection in humans. Previous studies on ticks as vectors of pathogens, carried out in Lower Slesia, were focused mainly on the presence of spirochetes of *Borrelia burgdorferi* s.l., regarded as the etiological agent of Lyme borreliosis (Kiewra, 2005; Kiewra et al., 2002, 2006). Lyme borreliosis is still considered the most common tick-borne disease in the Northern Hemisphere (Stanek et al., 2012). However *I. ricinus* can harbour also other pathogens (including bacteria, viruses and protozoa) (Franke et al., 2013). Human granulocytic anaplasmosis (HGA) has been recently recognized as a tick-borne emerging disease, which is spread over USA, Europe and Asia. In Europe, the first confirmed case of HGA was reported in 1997, in Slovenia (Petrovec et al., 1997), whereas the first cases of acute human granulocytic ehrlichiosis were described in 2001 in Poland (Tylewska-Wierzbanowska et al., 2001). It is worth noting, that *A. phagocytophilum* infection can be asymptomatic, and many infections can be unrecognized (Dumbler et al., 2005).

In Poland, questing *I. ricinus* ticks infected with *A. phagocytophilum* were found in north, north-eastern and central-eastern parts of the country (Stańczak et al., 2004; Grzeszczuk et al., 2002, 2004; Tomasiwicz et al., 2004; Wójcik-Fatla et al., 2009; Sytykiewicz et al., 2012). During present studies, the observed abundance of questing ticks in all examined localities in Lower Slesia, and also the infection of *I. ricinus* with *A. phagocytophilum* in Legnica, Milicz, Luban, and Oława districts, has revealed for the first time the risk for HGA transmission in SW Poland. The prevalence of infected *I. ricinus* with *A. phagocytophilum* was 4.3%. Similar level of infections, varying between 2% and 6%, depending on the area, were recorded in Lower Saxony, Germany (Templin et al., 2013), whereas the one in urban areas of Kosice, Slovakia reached 2.2% (Vichova et al., 2013), and 2.6% in Belarus (Reye et al. 2013). As many as 8.7% questing *I. ricinus* were found to be infected with *A. phagocytophilum* in north-eastern Poland (Grzeszczuk et al., 2004), 8.5% in central-eastern Poland (Sytykiewicz et al., 2012), 13.1% in mid-eastern Poland (Tomasiwicz et al., 2004), 14% in northern Poland (Stańczak et al., 2004). During the present studies no significant differences in infection rates between the districts were observed. However, the infection prevalence can depend on a study area and it may also vary depending on the year of study (Grzeszczuk and Stańczak, 2006a).

A. *phagocytophilum* has been found not only in questing ticks but also in ticks removed from human skin. For example, 23.7% *I. ricinus* ticks removed from patients in north-eastern Poland were infected (Grzeszczuk and Stańczak, 2006b). A high prevalence of *A. phagocytophilum* was also detected in engorged ticks from potential reservoir hosts such as roe deer (Overzier et al., 2013). The studies from the territory of Poland have confirmed that roe deer are essential hosts for maintaining *Ixodes* ticks and tick-borne diseases, including *A. phagocyto­philum* (Welc-Falęciak et al., 2013).

In our study, the percentage of infected females (15.2%) was almost 4 times higher than in the males (3.9%). Females were found to be more infected than males also in other parts of Poland (Tomasiwicz et al., 2004; Grzeszczuk, 2006; Grzeszczuk et al., 2002, 2004; Stańczak et al., 2004; Sytykiewicz et al., 2012). A significantly lower positivity rate (1.3%) was recorded in nymphs compared to adults. Also other authors
(Tomasiewicz et al., 2004; Grzeszczyk, 2006; Grzeszczyk et al., 2002, 2004; Stańczak et al., 2004; Szytkiewicz et al., 2012) have shown that the nymphs are less infected than adult ticks. The observed tendency may point to the limited role of small mammals in maintaining A. phagocytophilum in examined districts, compared to large-sized mammals. In Europe, A. phagocytophilum strains associated with rodents are transmitted by I. trianguliceps, and not by I. ricinus ticks (Pangracova et al., 2013). Thus, the occurrence of I. ricinus is not sufficient to rodent infection with A. phagocytophilum. However, I. ricinus can acquire A. phagocytophilum from large-sized mammals like cervids or wild boars, which can play a role as reservoir hosts (Michalik et al., 2009, 2012).

The obtained results confirm that I. ricinus ticks infected with A. phagocytophilum are widespread in Poland and capable of maintaining the A. phagocytophilum circulation in nature. Thus, in diagnosing the tick-borne disease in SW Poland, the possibility of infection with A. phagocytophilum should be also taken into account.

Acknowledgments
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Literature


Introduction

Crop productivity losses due to diseases can result in hunger and starvation especially in developing countries and soil borne fungi are the main causal agents decreasing crop productivity. At present, 1258 different fungal species including *Rhizoctonia solani* have been reported to cause these diseases or are potential threats to crop failure (Ciesielski *et al.*, 2009; Consolo *et al.*, 2012; Suwannarach *et al.*, 2012).

*R. solani* is a universal fungal pathogen, causal agent of plant roots and lower stem diseases. In Pakistan, *R. solani* occurs as subterranean forms; therefore, chemical control is not a viable choice until the availability of highly selective and efficient fungicides. Control measures of *R. solani* diseases are limited due to wide range of hosts and unavailability of resistant plant varieties (Rouf, 2002). Different strategies to control soil borne pathogens have been hypothesized. Amongst these, biological control has got the attention of most researchers (Benítez *et al.*, 2004; Vinale *et al.*, 2008; Consolo *et al.*, 2012; Chakraborty *et al.*, 2013). A large number of soil fungi have been known as potential biological control agents and among them *Trichoderma* exhibits the ability to control the plant pathogens (Punja and Utkhede, 2003; Ting and Choong, 2009; Chaudhary *et al.*, 2012). *Trichoderma* are the fast growing filamentous deuteromycetes found in a variety of soils. Due to effective biocontrol abilities of *Trichoderma*, many of its commercial biocontrol products are being marketed in Asia, Europe and USA but none of these are commercially available in developing countries like Pakistan (Consolo *et al.*, 2012).

The mechanisms involved in the biocontrol activity of *Trichoderma* spp. against plant pathogens are important in designing effective and safe biocontrol strategies (Wolska *et al.*, 2012). Different proposed mechanisms include: mycoparasitism (attack and killing of pathogen) (Anees *et al.*, 2010) and competitive inhibition for space and nutrients (Benítez *et al.*, 2004). *Trichoderma* are also known to produce different antibiotic substances e.g. gliotoxin, gliovirin, viridin, and trichoviridin (Vinale *et al.*, 2008). *Trichoderma* have also been known to inhibit the growth of pathogenic fungi by modifying the rhizosphere (Harman *et al.*, 2004). Moreover, infestation of *Trichoderma* in the rhizosphere helps plant to promote nutrient/fertilizer uptake (Yedidia *et al.*, 2003), seed germination and photosynthetic rates (Shoresh *et al.*, 2010).

F. S. Saeed Ahmad *et al.*

Biocontrol Efficacy of Different Isolates of *Trichoderma* against Soil Borne Pathogen *Rhizoctonia solani*

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Abstract

In this study, the biocontrol abilities of water-soluble and volatile metabolites of three different isolates of *Trichoderma* (*T. asperellum, T. harzianum* and *Trichoderma* spp.) against soil borne plant pathogen *Rhizoctonia solani* were investigated both in vitro and in vivo. The results showed for the first time that mycelial growth inhibition of the pathogen was 74.4–67.8% with water-soluble metabolites as compared to 15.3–10.6% with volatile metabolites in vitro. *In vivo* antagonistic activity of *Trichoderma* isolates against *R. solani* was evaluated on bean plants under laboratory and greenhouse conditions. We observed that *T. asperellum* was more effective and consistent, lowering disease incidence up to 19.3% in laboratory and 30.5% in green house conditions. These results showed that three isolates of *Trichoderma* could be used as effective biocontrol agents against *R. solani*.

Key words: *Rhizoctonia solani*, *Trichoderma*, antagonistic activity, biocontrol, soil born pathogen

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To our knowledge, no work has been carried out so far to explore the biocontrol abilities of indigenous *Trichoderma* populations. The current research was aimed at isolating the indigenous *Trichoderma* spp. and gauging their biological control potential against soil born plant pathogen *R. solani*. Both in vitro and in vivo trials were carried out to investigate different mechanisms involved in antagonistic activity of *Trichoderma* species. Furthermore, the suppression of disease incidence and related effects on growth were also observed in bean plants.

**Experimental**

**Materials and Methods**

**Fungal strains.** Three *Trichoderma* strains were isolated from agricultural soils as well as obtained from the Fungal Culture Bank of the University of the Punjab Lahore, Pakistan. A highly virulent strain of *R. solani* was isolated from infected bean plants. These fungal strains were maintained at 4°C on Potato Dextrose Agar (PDA) Merck, USA) with periodic sub-culturing on the same medium at 25°C.

**Molecular identification of *Trichoderma* strains.** The *Trichoderma* isolates were identified according to the protocol of Komoń-Zelazowska *et al.* (2007). DNA isolation was carried out according to Castle *et al.* (1998). The extraction of DNA was done with NucleoMag 96 Plant Kit (Macherey Nagel, Switzerland) and King Fisher technology (Thermo, UK). The primer sequences were; **EF1:** 5′-ATGGGTAAGGA(A/G)GACAAGAC-3′ and **EF2:** 5′-GGA(G/A)GTACCAGT(G/C)ATCAT-3′. The DNA was quantified by using the Nano drop 1000 (Thermo Scientific, Milan, Italy). For each sample, 1 µl of DNA (50 ng/µl) was amplified and the mixture (20 µl) contained 1 µl of 10x buffer, 0.5 mM of deoxynucleotide triphosphate each, 1 U Taq DNA polymerase (Qiagen, USA), 0.5 mM of each primer and 1.5 mM MgCl2. The PCR program was run as: 95°C, 3 min, 95°C, 1 min; 60°C, 1 min; 72°C, 3 min, 72°C, 5 min for 35 cycles. Five µl of PCR product was run on 1.5% agarose gel containing 1 µl DNA stain SYBR Safe (Invitrogen, USA) in 1x TAE buffer at 3.3 V for 30 minutes and images were obtained with Gel Doc 1000 System (Biorad Lab., USA). Purification of PCR products was done by QIA quick PCR Purification Kit (Qiagen, Milano, Italy) and sequencing was done by Deoxy terminator cycle sequencing kit (Perkin-Elmer Applied Biosystems) by BMR Genomics (Padova, Italy).

Homology of the sequences with other deposited nucleotide sequences was checked using basic Blast search program at NCBI and submitted to the website for *Trichoderma* species identification http://www.isth.info/tools/blast/index.php.

**Biocontrol efficacy of *Trichoderma* against *R. solani* in Dual Culture Assay.** A 5 mm plug of *Trichoderma* and *R. solani* was cut and incubated at 25°C as described in previous section 2.3. The control plate contained only *R. solani*. The mycelial growth of *Trichoderma* and *R. solani* was recorded after every 24 h, taking the radial growth at right angle to each other and calculating the average (Dennis and Webster, 1971a). Mycoparasitic activity was observed by using light microscope (Axioskop, Germany).

**Biocontrol efficacy of water-soluble metabolites of *Trichoderma* against *R. solani*.** PDA plates containing cellophane paper were inoculated with 5 mm mycelial discs of 3 days old cultures of *Trichoderma* isolates and incubated at 25°C for 3 days. After 3 days cellophane paper was removed and a 5 mm disc of pathogen was placed on the same PDA plate (Dennis and Webster, 1971b). The control treatment contained only pathogen disc grown without cellophane paper. The cultures were further incubated at 25°C until the colony of pathogen was spread on whole Petri plate in control treatment. The mycelial inhibition of pathogen by *Trichoderma* isolates was calculated using the Eqn. 1 (Edington *et al.*, 1971).

Mycelial Inhibition % = [(C2 − C1)/C2] × 100 (Eqn. 1)

Where, C1 = radial mycelial growth of *R. solani* in the presence of *Trichoderma* and C2 = radial mycelial growth of *R. solani* in control.

**Biocontrol efficacy of volatile metabolites of *Trichoderma* against *R. solani*.** The PDA plates were inoculated with 5 mm mycelial discs of 3 days old growing culture of *Trichoderma* isolates. The lid of each plate was replaced with the bottom of other plate inoculated with 5 mm mycelial discs of pathogen. Both plates were sealed together with adhesive tape and incubated at 25°C (Dennis and Webster, 1971c). Control treatment did not contain *Trichoderma* isolate. The mycelial inhibition of pathogen was calculated using Eqn.1.

**In vivo biocontrol activity of *Trichoderma* species on bean plants.** The bean plants were managed in growth chambers with 12 h photoperiod, 60% humidity and 25°C temperature. The inoculum was prepared according to Pugliese *et al.*, (2008). The *Trichoderma* isolates and pathogen were propagated on sterilized wheat kernel medium (75 g wheat kernel/80 ml H2O).
and incubated at 25°C in the dark for 10–15 days. *Trichoderma* strains at 5 g/l of inoculum were added to the plastic bags containing steam disinfected peat. Seven days after treatment, the substrate was infested with pathogen at 0.5 g/l and stored at 25°C in growth chambers. The soil of each bag was then transferred to one litre volume pots (10 × 10 × 12 cm) and bean seeds were sown at 5 seeds l⁻¹ of peat substrate. The pots were irrigated on a daily basis with sterilized water. 

Commercial formulations of *Trichoderma harzianum* ICC 012 2.00% and *Trichoderma viride* ICC 080 2.00% (Remedier®, Isagro Italia Milan, Italy) were used to verify the efficacy of *Trichoderma* isolates. A series of samples treated with a fungicide (Tolclofos-methyl) at the time of sowing of bean seeds were maintained as a fungicide (Tolclofos-methyl) was applied at 5 and 1 g/l of peat soil in plastic bags described in previous section.

Germination was completed five days after sowing and occurrence of any kind of disease was recorded. The plants were uprooted and washed with water. The roots were categorized using scale 0–4 where 0 = healthy plant (no infection), 1 = 25% infected root, 2 = 50% infected root, 3 = 75% infected root, 4 = 100% infected or completely dead plants depending on the appearance of elongate, sunken, red-brown lesions on roots and stems above or below the soil. The Disease index (DI %) was calculated according to Eqn. 2.

\[
DI\% = \frac{[(n \times 0) + (n \times 0.25) + (n \times 0.5) + (n \times 0.75) + (n \times 1)]}{N \times 100} \text{ (Eqn. 2)}
\]

Where, \( n \) = number of plants corresponding to each class, \( N \) = total number of plants observed. The *Trichoderma* isolates were also assessed for their effect on the growth of bean plants.

**Effect of inoculum dose on in-vivo biocontrol activity against *R. solani*.** The antagonists and the pathogen were grown on wheat kernel medium as described in previous section. *Trichoderma* isolates were applied at 5 and 1 g/l of peat soil in plastic bags and the bags were kept at green house conditions for one week. After one week, each bag was inoculated with *R. solani* at 0.25 g/l of soil. The soil of each bag was then transferred to two litres volume pots (12 × 12 × 14 cm) and seeds were sown at 10 seeds/pot. Controls were set up along each treatment.

**Experimental layout and Statistical analyses.** The experiments were set up in randomized complete block design with four replicates for each treatment. Statistical analysis was carried out using SPSS (version 17.0 ChicagoIL, USA). Analysis of variance (ANOVA) was performed at 5% significance level. Duncan's HSD multiple range test was used as post-hoc analysis to compare means. Pearson's correlation coefficient was calculated to analyze the effect of disease incidence on fresh biomass of bean plants.

**Results**

**Molecular identification of *Trichoderma* species.** *Trichoderma* isolates were identified on the basis of 18S RNA gene sequencing with amplification of *tef1* domain at 5’ end. The sequences were compared with other nucleotide sequences at NCBI databases using Basic Local Alignment Search Tool (BLAST) and were submitted to Gene Bank (Bankit) for accession numbers. The amplicon of *Trichoderma* TV showed a 99% homology (808/809 and 806/808 bp) with the nucleotide sequence of *T. asperellum* Th021 (AB568376.1) and *T. asperellum* Th016 (AB568375.1) respectively while, *Trichoderma* TK showed 99% homology (738/739 and 738/739 bp) with nucleotide sequence of *T. harzianum* strain CIB T127 (EU279980.1) and *T. harzianum* strain DAOM 167671 (AY605783.1) respectively. The identification was confirmed by searching *tef1* sequences by *Tricho BLAST*.

**Growth profile of *Trichoderma* species at different pH and temperature.** At 25°C and 30°C, the three fungal species (*T. asperellum, T. harzianum* and *Trichoderma* spp.) showed maximum mycelial growth while, at 20°C the growth rate was considerably reduced and antagonists colonized 1/4th of the medium surface. In acidic pH range *i.e.*, 5–6, the mycelial growth was maximum whilst, moderate growth was observed at pH 6.5 and 7.0 by antagonists. Beyond these pH limits no growth or very little growth (0.9–1.2 cm) was recorded.

**Biocontrol efficacy of *Trichoderma* against *R. solani* in dual culture assay.** The results demonstrated a strong antagonistic potential of *Trichoderma* against pathogen (Fig. 1). A clear zone of interaction between antagonist and pathogen was observed where the former inhibited the growth of later after making a physical contact. Light microscopic analysis further revealed a typical coiling pattern of *Trichoderma* species around the hyphae of *R. solani* (Fig. 2). This hyphal interaction was initiated after 72 h of incubation. After seven days of incubation, pathogen hypha started to disappear and *T. asperellum, T. harzianum* and *Trichoderma* spp. completely overgrew the pathogen.

**Biocontrol efficacy of water-soluble and volatile metabolites of *Trichoderma* against *R. solani*.** The water-soluble metabolites of all the *Trichoderma* isolates proved to be considerably effective in limiting the growth of *R. solani*. Growth inhibition was significantly higher (p < 0.01) with *T. asperellum* (74.4%) followed by *Trichoderma* spp. (70.0%) and *T. harzianum* (67.8%) as compared to control treatment for water-soluble
metabolites (Table I). All *Trichoderma* isolates exhibited growth inhibition of less than 20% for volatile metabolites (Table I). The values were 15.3% for *T. harzianum*, 11.8% for *T. asperellum* and 10.6% for *Trichoderma* spp. compared to control treatment.

**In vivo biocontrol activity of Trichoderma species against *R. solani***. The results showed that *T. asperellum* was the most effective biocontrol agent at all application times as it associated with the lowest disease incidence. The relative fresh biomass production was increased compared to inoculated control (p < 0.01). When the *Trichoderma* was applied seven days before inoculation of pathogen, all species showed an elevated (19.3 to 26.3%) biocontrol efficacy against *R. solani* compared to inoculated control (54.3%) (Table II). Among the three isolates, *T. asperellum* showed maximum efficacy by lowering the disease incidence up to 19.3% when disease index was 54.3% in inoculated control (p < 0.01) (Table II). However, the disease incidence was 23.3% by *T. harzianum* and 26.3% in case of *Trichoderma* spp. Commercial formulation, Remedier®, proved to be less effective (p < 0.01) than the tested *Trichoderma* species with a disease incidence of 38.3%. In addition, relative biomass (RW) was decreased as a consequence of pathogen infection (Table II).

All isolates proved to have a positive effect on the growth of bean plants yielding a higher biomass compared to healthy control ones. *T. asperellum* yielded highest relative biomass of 127% (p < 0.01) compared to healthy control, followed by *T. harzianum* providing a relative biomass of 113%. *Trichoderma* spp. provided lowest relative biomass (107%) among the species (Table II).

In another experiment, where the antagonist was applied seven days after the pathogen, the best results were observed with *T. asperellum* with 19.7% disease incidence compared to inoculated control (53.3%). This antagonistic behavior was correlated with higher relative biomass *i.e.*, 168% relative weight. Similarly, *T. harzianum* exhibited disease suppression of 23.2%, while the relative biomass was enhanced up to 155%. However, *Trichoderma* spp. showed a disease incidence of 21% but the effect on relative weight of bean plants was low (94%). The commercial formulation, Remedier® showed least biocontrol efficacy (27.7%) and lowest relative biomass (74%) compared to all tested isolates (Table II). When the *Trichoderma* species were evaluated for growth promoting ability, a positive effect on the relative biomass of bean plants was observed. Among the species, *T. harzianum* yielded a highest

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**Table I**

Effect of water-soluble (A) and volatile (B) metabolites of *Trichoderma* on growth inhibition of *R. solani*.

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Mycelial growth of <em>R. solani</em> (cm)*</th>
<th>% mycelial inhibition at 72 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 h</td>
<td>48 h</td>
</tr>
</tbody>
</table>

(A)

| *T. asperellum* | 0.9 ± 0.07 | 1.0 ± 0.14 | 1.2 ± 0.07 | 74.4** |
| *T. harzianum*  | 0.8 ± 0.14 | 1.2 ± 0.07 | 1.5 ± 0.07 | 67.8 b |
| *Trichoderma* spp. | 1.0 ± 0.07 | 1.1 ± 0.07 | 1.4 ± 0.07 | 70.0 b |
| Control (R. Solani) | 1.8 ± 0.14 | 3.1 ± 0.00 | 4.5 ± 0.00 | 0.0 c |

(B)

| *T. asperellum* | 1.2 ± 0.00 | 2.4 ± 0.14 | 3.8 ± 0.07 | 11.8 b** |
| *T. harzianum*  | 1.2 ± 0.07 | 2.5 ± 0.21 | 3.6 ± 0.00 | 15.3 a |
| *Trichoderma* spp. | 1.2 ± 0.00 | 2.4 ± 0.28 | 3.8 ± 0.00 | 10.6 b |
| Control (R. Solani) | 1.3 ± 0.14 | 2.5 ± 0.07 | 4.3 ± 0.07 | 0.0 c |

* Values are means of four replicates of two independent experiments ± SE.
** Values followed by the different letters in the column are statistically different by Duncan’s HSD multiple range Test (p < 0.05).
Biocontrol efficacy of *Trichoderma* sp. against *R. solani*. 1999 relative biomass of 126% while, *T. asperellum* helped in providing 114% of relative biomass. *Trichoderma* spp. did not show any improvement producing 100% relative biomass (Table II).

**Effect of inoculum dose of Trichoderma species on in vivo biocontrol activity against *R. Solani***. All the *Trichoderma* species showed a higher control efficacy both at high and low dosages, compared to inoculated control. *T. asperellum* was more effective in both trials and showed a decrease in disease incidence with increase in concentration of antagonist, providing a control efficacy of 29.1 and 35.3% (p < 0.01), when applied at a dose of 5 and 1 g/l in first trial respectively. However, *Trichoderma* spp. and *T. harzianum* did not show significant differences among the treatments with high and low doses in first trial. In second trial, *T. asperellum* provided the lowest disease incidence but no statistical differences among treatments with dosages (p < 0.01), while *Trichoderma* spp. was more effective at a dose of 5 g/l with an efficacy of 35.1% (p < 0.01), compared to 39.0% disease incidence with 1 g/l dosage. The *T. harzianum* did not show significant difference in the disease incidence in both trials applied at both dosages. Remedier®, showed a disease index of
48.8 and 34.8% in first and second trial respectively (Table III). Chemical treatment was effective in controlling the disease with an efficacy of 34.4 and 20.0% (p < 0.01) in the first and second trial respectively. All the treatments without pathogen showed a high biomass than health control (p < 0.01). The \textit{T. asperellum} showed maximum biomass production of 61.2 g in first trial when 5 g/L dose was applied while in second trial the \textit{Trichoderma} spp. yielded a maximum biomass of 60.5 g with same dose. In the treatments with pathogen, the above ground biomass was reduced compared to health control due to consequence of disease (Table III).

The chemical treatment with tolclofos-methyl showed the highest disease suppression and was more effective than the tested fungal strains. The chemical treatment showed 16.7–17.7% disease incidence with
both types of experimental setup. A weak negative correlation was observed for the relative biomass production and disease incidence on bean plants when infested with both pathogen and Trichoderma species \((r = -0.308)\). Moreover, the fresh biomass production of bean plants was negatively correlated \((r = -0.538)\) with disease incidence (Table IV).

### Discussion

The present study evaluated the biocontrol efficacy of three indigenous strains of Trichoderma isolated from agricultural land in Pakistan against soil born plant pathogen R. solani.

Two Trichoderma isolates out of three used in the present study were identified on the basis of 18S rRNA and they were identified as T. asperellum and T. harzianum. Both species were previously identified as efficient biocontrol agents against several plant pathogens (Schuster and Schmoll, 2010). Likewise, these isolates showed considerable biocontrol efficacy against R. solani in in vitro and in vivo experimental conditions.

Trichoderma species are distributed worldwide in the rhizospheric regions of plants and around decaying dead biomass (Kubicek et al., 2008). They are extraordinarily able to adjust to the surrounding environmental conditions by regulating their metabolism, growth and reproduction (sporulation). The pH and temperature proved to be major limiting factors affecting the growth profile of Trichoderma (Schmoll et al., 2010). The isolates in the present study showed maximum growth at 25°C under acidic pH ranging from 5–6 which establishes their mesophilic nature. These findings are in agreement with Hajieghrari et al. (2008); where optimum pH varied from 5–8 and temperature from 25–30°C among different species of Trichoderma.

For the control of plant pathogenesis, Trichoderma species (mycoparasitism) (Vinale et al., 2006) and/or their extracellular metabolites can be exploited as biocontrol agents or biological fungicides. These metabolites include; volatile and water-soluble metabolites (Eziashi et al., 2006) and secondary metabolites of low molecular weight (Schuster and Schmoll, 2010). The Trichoderma isolates studied were not only able to inhibit the growth of pathogen in in vitro experiments (Fig. 2, Table I) but also capable of suppressing the disease incidence by the pathogen in in vivo trials (Table II, III) confirming their versatile defensive mechanisms. In this context T. asperellum proved to be the most effective among the tested isolates. T. asperellum has been identified as a potential biocontrol agent in other studies (Osorio-Hernandez et al., 2011) where it showed in-vitro inhibition of pathogen in the range of 11–16%. Viterbo et al. (2005) characterized a protein kinase TmkA from T. asperellum, which had a key role in the regulatory pathways involved in biocontrol activity. In vitro studies revealed that Trichoderma had comparatively higher growth rates which provide them competitive advantage over the pathogen in availing space and nutrients in the medium. These species also inhibited the growth of the pathogen by secreting certain mycotoxins (Cundom et al., 2003).

Mycoparasitism is one of the major activities occurring in the antagonist-pathogen interaction, expressed in different steps in a sequence. The detection, attachment, direct penetration, and secretion of fungitoxic enzymes which leads to death of pathogen are major actions in their interface (Harman et al., 2004). The interactions of Trichoderma and pathogen in dual culture were observed under light microscope and the hyphal contact between Trichoderma and pathogen started after 48–72 h of incubation. Once the contact was established, dense hyphal coiling of Trichoderma around R. solani hypha, a characteristic response of antagonists was prominent. Similar observations were previously noted against R. solani by Almeida et al. (2007). The antagonists showed an affinity for the host cell wall which suggests that this may involve chemical bonding between functional sites of carbohydrates present on the cell wall of Trichoderma and pathogen which triggers the events leading to host wall penetration (Eziashi et al., 2007).

The production of antifungal compounds also play important role in antagonistic activity of Trichoderma species. These include; antibiotics, mycotoxins and low-molecular weight secondary compounds (Schuster and Schmoll, 2010). Our results indicated that all three isolates were able to produce water-soluble metabolites that inhibited the mycelia growth of R. solani. T. asperellum proved to be the highest producer of these metabolites, while the production of non-volatile metabolites

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**Table IV**  
Correlation between disease incidence and corresponding biomass of bean plant in different experiments.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Pearson's coefficient ((r))</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Trichoderma applied seven days before pathogen (laboratory)</td>
<td>-0.538</td>
<td>(p = 0.01)</td>
</tr>
<tr>
<td>2. Trichoderma applied seven days after pathogen (laboratory)</td>
<td>-0.308</td>
<td>(p = 0.08)</td>
</tr>
<tr>
<td>3. Effect of concentration on biocontrol efficacy of Trichoderma (greenhouse)</td>
<td>-0.843</td>
<td>(p = 0.00)</td>
</tr>
</tbody>
</table>
was not obvious. Therefore, the principle mechanism of antagonistic activity against pathogen was speculated as mycoparasitism (Eziashi et al., 2007) and antibiosis or due to the production of secondary metabolites as suggested by Howell (2003). These speculations were supported by the suppression of disease incidence by all Trichoderma isolates in in vivo trials on bean plants. An increased biocontrol efficacy compared to other isolates and control treatments was provided by T. asperellum in terms of application of antagonists before and after the incorporation of pathogen in the soil.

Results in the present study also indicated that the inoculation of antagonist seven days before the pathogen was more effective. These results are in line with De Figueiredo et al. (2010) who studied the actions of Trichoderma against Sclerotinia sclerotiorum in bean plants. The pathogenicity was found to be reduced to 37.04% when antagonist was applied eight days before the pathogen. This approach was also recommended by Lewis and Lumsden (2001).

Interestingly, Trichoderma isolates proved to be more effective in controlling the R. solani than the commercial formulation Remedier®. This indicates that it is not necessary to apply Trichoderma species in complex formulations (Harman, 2000). Additionally, the single strain of Trichoderma can be considerably capable of controlling diverse pathogens. Perhaps it would not be possible to commercialize the mixture of biocontrol strains unless there is highly significant success in biological control.

Trichoderma species are well known for their abilities to promote plant growth by colonizing the roots of plants. Their interactions of antagonists with plants enhance the root proliferation and yield production by increasing uptake of nutrients (Harman et al., 2008). The fresh biomass of the bean plants was increased up to 118% when treated with the Trichoderma isolates in greenhouse trials as compared to health control (Table III), which significantly proved the ability of Trichoderma as a plant growth promoter. These findings are similar to those reported by Pugliese et al. (2008) where Trichoderma isolates controlled R. solani and increased the biomass of bean plants up to 163%. Likewise antagonists prevented 100% mortality of tomato plants coupled with an increase in plant fresh and dry weight (Montealegre et al., 2010). Shaban and El-Bramawy (2011) investigated the biocontrol of damping-off and root rot diseases by combining Trichoderma spp. and Rhizobium species. They reported an overall improvement in plant growth, seed and fruit production. In the present study, the different isolates of Trichoderma showed as an effective biocontrol agents against R. solani though their efficacy varied among isolates and it was highest with T. asperellum. The biocontrol efficacy of all Trichoderma isolates was even higher than commercial formulation Remedier® in in vivo trials. Apart from suppressing the disease profile of bean plants, these isolates showed a considerable effect in promoting their general growth.

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Literature


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ORIGINAL PAPER

Introduction

Epstein-Barr virus belongs to the Herpesviridae family, Gammaherpesviridae subfamily, Lymphocryptovirus genus and is also known as human herpesvirus 4 (HHV-4). Herpesviruses have double-stranded DNA genome (Peh et al., 2003). EBV is an enveloped virus with an icosahedral capsids symmetry. The genome takes on a linear form in mature virions and a circular episomal form during the period of latency in the infected cells. Epstein-Barr virus express six nuclear proteins: EBNA (EBV nuclear antigens) which include EBNA-1, EBNA-2, EBNA-3A, EBNA-3B, EBNA-3C and EBNA-LP (leader protein), next are three latent membrane proteins LMP-1, LMP-2A, LMP-2B and two untranslated RNAs termed EBV-encoded RNA (EBER-1 and EBER-2). LMP-1 is a transmembrane protein, it is a major oncogenic protein of Epstein-Barr virus and EBV strain are categorized due to nucleotide base-pair (bp) changes in the LMP-1 gene. Del-LMP-1 (variant with deletion) has a greater tumorigenic potential thus being responsible for an increased risk of nasopharyngeal carcinoma (NPC), and is less immunogenic (Perera et al., 2010). This virus has the ability to replicate and enter a latency phase in B cells, which can lead to a development of viral types of cancer (Chen 2011). So far, two types of EBV (EBV-1 and EBV-2) have been distinguished, with the significant differences in the EBNA-2, EBNA-3A, EBNA-3B and EBNA-3C gene sequences (Peh et al., 2003, Correra et al., 2007, Tiwawech et al., 2008). Type 1 is found primarily in Europe, North and South America and Asia, while type 2 is mainly dominant in Africa(Peh et al., 2003). EBNA-2 protein plays a key role in initiation of carcinogenesis by disrupting mitotic checkpoints and causing chromosomal instability (Pan et al., 2009). EBV is an etiological agent of infectious mononucleosis also known as ‘the kissing disease’, because the virus spreads through direct mouth-to-mouth contact with saliva being the main

Prevalence of EBV Genotypes in Polish, Taiwanese and Arabic Healthy Students and Association Between Genotypes and 30-bp Deletion in the LMP-1 Gene

Phylogenetic Analysis

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Abstract

The aim of this study was to compare the prevalence of EBV genotype and del-LMP-1 in saliva from Polish, Taiwanese and Arabic healthy students. The study group consisted of 56 healthy students; 24 of them Polish, 25 Taiwanese, and 7 Arabic. Typing was carried out using PCR with EBNA-2 primers. A detection of LMP-1 variants was also performed using PCR. EBV DNA was detected in 22 investigated samples (39.3%). Type 1 of the virus was dominant in both Polish and Taiwanese group. Among 62.5% Tai wanese with EBV 1 and 55.6% Polish detected EBV with 30-bp deletion in LMP-1 gene.

Key words: EBV, saliva, LMP-1

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The investigated material was saliva. Samples were obtained from healthy students of the Medical University of Lublin, Poland. The study group consisted of 56 healthy volunteers: 24 Polish, 25 Taiwanese, and 7 Arabic students. In the study group there were 26 female and 30 male in 18–37 years of age (22.2 average). Saliva samples were collected anonymously, and all volunteers were healthy; there was also an a survey containing clinical about general and infectious diseases data attached. The research received approval from the Ethics Committee number KE-0254/150/2010.

**Experimental**

**Material and Methods**

**Sample collection.** The investigated material was saliva. Samples were obtained from healthy students of the Medical University of Lublin, Poland. The study group consisted of 56 healthy volunteers: 24 Polish, 25 Taiwanese, and 7 Arabic students. In the study group there were 26 female and 30 male in 18–37 years of age (22.2 average). Saliva samples were collected anonymously, and all volunteers were healthy; there was also an a survey containing clinical about general and infectious diseases data attached. The research received approval from the Ethics Committee number KE-0254/150/2010.

**DNA extraction.** DNA from 200 µl of saliva samples was extracted using QIAamp DNA Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. All PCR reactions were carried out in final volume of 25 µl using HotStartTaq DNA Polymerase (Qiagen, Germany) according to the manufacturer's instructions. Concentrations of PCR reaction components were prepared as follows: 2.0 mM MgCl₂ (Qiagen, Germany). Concentrations of PCR reaction components were prepared as follows: 2.0 mM MgCl₂, 0.2 mM dNTPs, 0.5 µM each forward and reverse primers and 0.5 U of HotStart Taq polymerase. During each run samples were tested together with one negative and positive control.

**Amplification of EBNA-2 gene.** The nested PCR was carried out for amplification of EBNA-2. The sequence of primers used for PCR was as follows: outer pair 5’-TTT CAC CAA TAC ATG ACC C-3’, 5’-TGG CAA AGT GCT GAG AGC AA-3’ and inner pair 5’-CAA TAC ATG ACG CRG AGT CC-3’, 5’-AAG TGC TGA GAG CAA GGC MC-3’, (R = A/G, M = A/C). 2 µl of extracted DNA was subjected to the PCR mixture with the concentration as described above. The first-round amplification consisted of activation of polymerase 95°C for 15 minutes, 35 cycles of 94°C for 1 minute, 55°C for 1 minute, 72°C for 2 minutes and the final extension at 72°C for 5 minutes. The second-round amplification was performed with 1 µl of first round PCR product in 30 cycles with an annealing temperature at 60°C. The amplicons 368 bp, 473 bp in length (depending on the EBV type EBV-1 and EBV-2, respectively) were separated on 2% agarose gel and purified using Gel- Out kit (A&A Biotechnology, Poland) for further analysis. Purified PCR products were send to Genomed Warsaw, a company dealing with the field of genomics.

**Amplification of LMP-1 gene.** Primers used for the amplification were 5’-AGCGACTCTGCTGGAAATGAT-3’ and 5’-TGATTAGCTAAGGATTCCCA-3’. The reaction mixture containing 3 µl of extracted DNA was amplified under following conditions: 95°C for 15 minutes of initial hot start activation, then 40 cycles of 94°C for 1 minute, 57°C for 1 minute, 72°C for 1 minute with the final extension at 72°C for 10 minutes. The PCR products were subjected to 3% agarose gel and LMP-1 variants (316 bp – wild type or type with 30-bp deletion) were analyzed.

**Phylogenesis of EBV (phylogenetic analysis).** The results of sequencing were analyzed using the following computer programs: Chromas Lite 2.0.0.0, ClustalX 2.1.0.0, GeneDoc 2.7.0.0 and BioEdit 7.0.9.1. Typing was performed using the BLAST algorithm (basic Local Alignment Search Tool; http://blast.ncbi.nlm.nih.gov/BLAST.cgi). EBNA-2 gene sequence were used in order to construct phylogenetic tree. Phylogeny was based on the maximum likelihood method (ML). This method requires the use of computer programs such as PAUP 4.0, ModelTest 3.7, PhyML 2.4.4 and MEGA 4.1. In order to choose the appropriate model of molecular evolution hLRTs (hierarchical Likelihood-Ratio Test) and AIC (Akaike Information Criterion) test were used. The reference strains sequences used in the study was taken from the public database GenBank (http://www.ncbi.nlm.nih.gov/genbank/).

**Statistical analysis.** PQ Stat 1.4.6. program was used for the statistical analysis, and the relationship between investigated parameters was verified by means of V-Cramer test. Statistical significance was defined as P < 0.05.

**Results**

EBV DNA was detected in 39.3% (22) investigated samples, EBV type 1 was detected in 18 cases (81.8%) and EBV type 2 was detected in 4 cases (18.2%). The findings revealed that more Taiwanese students was EBV-positive compared to Polish and Arabic students (44%, 37.5% and 28.6% respectively). Type 1 of the virus was dominant in both Polish and Taiwanese group (100% and 72.7% respectively). The phylogenetic tree and percentage similarity of EBNA-2 gene sequences are shown in Figure 1 and in Table I. In the group of Arabic students EBV was detected in 2 cases out of seven respondents, amounting to 26.8 %. The low number of EBV-positive representatives enables an interpretation of this result (1 case of EBV1 and EBV2 was detected).

Among 54.5% Taiwanese and 55.6% Polish students EBV with 30-bp deletion in LMP-1 gene was detected.
Discussion

Saliva is a very interesting diagnostic material, sometimes described as the "mirror of the body". The undeniable benefit of this material is the way of obtaining samples; non-invasive nor stressful for the patient (especially when compared to collecting blood samples), the risk of needle stick is eliminated and at the same the risk of infection reduced. What is more, if blood is not visible in the saliva, the sample is not considered a class II biohazard (according to the US Centre for Disease Control) which also provides safety benefits for researchers. It is a painless method, which is of particular importance especially in the diagnosis of children. In addition, because of the way of collecting samples, it is inexpensive and the medical personnel does not require specialized training. Also the method of storing and preparing the material for further research is less complicated than in the case of serum (blood) (Shirtcliff et al., 2001). Saliva is used in the diagnosis of hormonal disorders (determination of cortisol concentration), it is considered to be good material for detecting oral cancer markers and monitoring the course of disease: the main biomarker is the tumour-suppressor protein TP53. Mutations in the p53 gene are considered to be the most common changes found in cancer, including squamous cell cancer of the oral cavity. Another cancer marker detected in saliva is CA125, used to diagnose ovarian cancer. Saliva as a diagnostic material has its application in virology: it is possible to detect anti-HIV IgM antibodies, using techniques of molecular biology (PCR) it is also possible to detect HSV, EBV, measles, and mumps (Farnaud et al., 2010).

EBV infection is transmitted from host to host via saliva, and the virus passes through the oropharyngeal epithelium to B lymphocytes (Thompson and Kurzrock 2004).

The Epstein-Barr virus belongs to a group of onco-genic viruses. The spectrum of diseases associated with being infected with this virus is very broad and the virus itself is common in the population. In our studies, the EBV DNA was detected in 39.3% of the students. Type 1 was detected in all of the Polish students (100%) while in the Taiwanese the detection of type 1 amounted to 62.5% (5 samples), so deletion in EBV 1 was more often detected among Taiwanese than among Polish students.
72.7%. 30-bp deletion in the LMP-1 gene was detected in 54.5% of Taiwanese and 55.6% of Polish students.

The results of our studies are consistent with the experiences of other authors e.g. higher incidence of EBV-1 in the healthy population is also confirmed by Correa et al. (2007).

LMP-1 EBV is a viral oncoprotein, which has an in vitro confirmation of the ability to transform cells and of inducing tumor growth (in vivo), it can disturb the growth and maturation of human keratinocytes and induce the expression of epidermal growth factor receptor connected to the nasopharyngeal carcinoma (NPC), and is probably associated with the tumor being more aggressive. Some researchers (Tiwawech et al., 2008 and Peh et al., 2003) report that the variant with deletion (del-LMP-1) is connected to changes which induce the protein’s oncogenic properties, Sandvej et al. (1994) combine the EBV-2 and del-LPM-1, suggesting the genotype transforming ability. There is little information on prevalence of del-LMP-1 in healthy individuals. In Brazil and Mexico this deletion was detected in 59% (Chen et al., 1996 , Dirnhofer et al., 1999), among healthy Italian blood donors in 44% (Dolcetti et al., 1997), in Argentina only in 7.4% (Correa et al., 2004). Tiwawech et al. (2008) present studies where EBV-1 is dominant (86.5–96%) in nasopharyngeal carcinoma (NPC) among Asians, and EBV-2 is less frequently detected (4–13%). They also confirm a strong correlation between a virus with deletion in LMP-1 and NPC in comparison to the wild-type gene (without deletion) and NPC. An increasingly interesting and still valid is the question why EBV causes NPC only in some populations and maybe the answer lies within the genetic differences between the strains. Tiwawech et al. (2008) indicate that type 1 (A) of the virus has a greater potential of transforming B-lymphocytes than type II (B).

The result of studies of Tse et al. (2009) and Bei et al. (2010) (genome-wild association study) indicate that the increased incidence of NPC among the Taiwanese and eastern China population are an outcome of genetic differences in the human leukocyte antigen (HLA) and multiple loci (HLA-A, HLA-F, GABBR1) within chromosome 6 p 21.3 is associated with this type of cancer.

Also Perera et al. (2010) indicate that the genetic differences between populations may influence the degree of risk of NPC occurrence, they provide the example of HLA-A11 restricted cytotoxic T-lymphocyte response is directed toward a specific epitope of the EBNA-4 EBV protein. They also confirm the reports on the influence of genetic alterations in virus strains, which determine the active infections in humans. Available literature does not provide studies comparing nationalities studied by us, it is worth to underline however that the study was conducted among healthy young people. Perhaps this is related to the significant differences in the incidence of this cancer. Significant differences in NPC incidence depending on the geographical location were observed; in south-east Asia (an area covering southern China, Hong Kong, Taiwan), the annual incidence rate is about 25 times higher than in the western world (Tse et al., 2009). It is believed that NPC in South East Asia is endemic, while the annual incidence rate for the rest of the world amounts to less than 1/100 000 (Ruan et al., 2013). The incidence rate of NPC in the Guandong province (China) is 30.94/100 000 for men and 13/100 000 for women, in Taiwan it is estimated at 6/100 000, being eighth among cancers leading to death. Mortality is 4/100 000 and mainly concerns middle aged patients (Lin et al., 2001, Ruan et al., 2013). Hong Kong recorded some of the highest rates of incidence: 4.8/100 000 for women and 15.0/100 000 for men (Paw et al., 2011). In Poland, this type of cancer is rare among both men and women. In 2010, the absolute number of men affected with nasopharyngeal carcinoma was 119, crude rate 0.6, standardized rate 0.4, percentage 0.2, and is in place 49 in sequence number with regards to the location of particular organs: In the same years there were 50 cases of women suffering from the nasopharyngeal carcinoma, crude rate 0.3, standardized rate 0.2, percentage 0.1, and is 67 in sequence number. NPC is so uncommon in Poland that there are no available data apart from those provided above ( Wojciechowska et al., 2010).

EBV DNA is detected in the majority of cases of NPC among Asians and about 75% of case among Caucasians (Pow et al., 2011).

Conclusions. As with other cancers, early detection is most significant for the prognosis. If the cancer is detected at an early stage, the patient’s chances of survival increase. In case of cancers with a viral pathogenesis it is worth to consider the possibility of virological testing. Although in the Polish population NPC is rare, nevertheless it is recorded and one should remember about the correlation NPC/EBV DNA. What is more, in the age of globalization, the number of patients from the most remote parts of the world is likely to increase, and thus the number of rarely recorded diseases may also increase.

In conclusion, the knowledge on EBV prevalence in healthy population of Polish students will be useful in analyzing the role of EBV and regional factors in the pathogenesis of EBV-associated cancers.

Literature


Sandvèj K., S.C. Peh, B.S. Andresen and G. Pallesen. 1994. Identification of potential host spots in the carboxy-terminal part of Epstein-Barr virus (EBV) BNLF-1 gene in both malignant and benign EBV-associated diseases; High frequency of a 39-bp deletion in Malaysian and Danish peripheral T-cell lymphomas. Blood. 84: 4053–4060


Microbial Transformations of 3-methoxyflavone by Strains of *Aspergillus niger*

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**Abstract**

Microbial transformation of 3-methoxyflavone into 3’-hydroxyflavon-3-yloxymethyl myristate was presented. Six filamentous fungi were used as biocatalysts: a wild strain of *Aspergillus niger* KB, its four UV mutants (*A. niger* MB, SBP, SBJ, 13/5) and the strain of *Penicillium chermesinum* 113. The highest yields were observed for the strains of *A. niger* KB and *A. niger* SBP (69.8% and 63.1%, respectively).

**Key words:** *Aspergillus niger*, *Penicillium chermesinum*, biotransformation, 3-methoxyflavone, myristic acid

Microbial transformation of flavonoid compounds is a natural method which may be a green alternative to chemical synthesis. Using biotransformation we can modify structures of compounds in order to improve their biological properties and to increase their hydrophilicity or bioaccessibility (Das and Rosazza, 2006; Wang et al., 2010).

Regioselective O-demethylation of tangeretin and 3-hydroxytangeretin by *Aspergillus niger* gave 4’-O-demethylated biotransformation products (Buisson et al., 2007) while transformations of 7,8-dimethoxyflavone by *Mucor ramannianus* gave five biotransformation products involving hydroxylation at C-3’ and C-4’ with the methoxyl groups retained and products of hydroxylation in the B-ring along with demethylation at C-7 or/and C-8 (Herath et al., 2009).

This manuscript reports the microbial demethylation of 3-methoxyflavone by strains of *Aspergillus* and *Penicillium*, followed by non-typical esterification with a fatty acid and hydroxylation in the B-ring.

The analytical procedures were as described previously (Kostrzewa-Suslow and Janeczko, 2012a; 2012b). The substrate for biotransformation – 3-methoxyflavone was purchased from Sigma-Aldrich, Poznań, Poland.

3-Methoxyflavone (C_{30}H_{38}O_{6}): M.p. 114–115°C. Rt 19.35 min (HPLC).

3’-Hydroxyflavon-3-yloxymethyl myristate (C_{30}H_{38}O_{6}): Yellow oily liquid. Rt 17.06 min (HPLC). Purity 97% (HPLC). HRESI-MS: m/z = 495.1032 [M+H]+; found 495.1026.

**1H NMR (DMSO-d_6)**: δ: 3.81 (3H, s, -OCH_3), 7.49 (1H, t, J_6,7 = 8.0), 7.58 (3H, m, H-3’, H-4’, H-5’), 7.75 (1H, d, J_6,7 = 8.2 Hz, H-8), 8.05 (2H, m, H-2’, H-6’), 8.10 (1H, dd, J_5,6 = 8.0, and J_5,7 = 1.5 Hz, H-5’); **13C NMR (DMSO-d_6)**: δ: 60.2 (-OCH_3), 119.0 (C-8), 124.1 (C-10), 125.4 (C-6), 125.6 (C-5), 128.8 (C-2’, C-6’), 129.2 (C-3’), 131.0 (C-4’), 131.4 (C-1’), 134.6 (C-7), 141.3 (C-3), 155.3 (C-2’), 155.5 (C-9), and 174.4 (C-4).

In this research we used a wild strain of *A. niger* KB and four UV mutants of *A. niger* (13/5, SBJ, SBP, MB). The KB strain comes from the collection of the Department of Biotechnology and Food Microbiology of Wrocław University of Environmental and Life Sciences (Poland), strain 13/5 comes from the University of Life Sciences in Lublin (Poland) and strains SBJ, SBP and MB come from Wrocław University of Economics (Poland). Microorganisms were maintained on sterilized potato slants at 5°C. The wild strain of *P. chermesinum* 113 was obtained from the collection of the Department of Chemistry of Wrocław University of Environmental and Life Sciences (Poland). The microorganism was maintained on Sabouraud 4% dextrose-agar slopes and freshly subcultured before use in the transformation experiments.

Screening tests and preparative-scale biotransformations were carried out according to the procedure described earlier (Kostrzewa-Suslow and Janeczko, 2012a; 2012b). The spectral data of the product obtained are presented below.

3’-Hydroxyflavon-3-yloxymethyl myristate (C_{30}H_{38}O_{6}): Yellow oily liquid. Rt 17.06 min (HPLC). Purity 97% (HPLC). HRESI-MS: m/z = 495.1032 [M+H]+; found 495.1026. **1H NMR (DMSO-d_6)**: δ: 0.86 (3H, t, J = 7.0 Hz, 13”-CH_3), 1.229–1.231 (14H, m, 1.229–1.231 (14H, m, H-4”-H-10”),

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1.239–1.261 (6H, m, H-3”, 11”, 12”), 1.55 (2H, tt, J = 7.67 Hz, H-2”), 2.25 (2H, t, J = 7.4 Hz, H-4”), 7.30 (1H, m, H-4’), 7.39 (1H, ddd, J = 7.9, J = 1.1 Hz, H-6), 7.49 (3H, m, H-8, 2’, 5’), 7.66 (1H, ddd, J = 8.4 Hz, J = 7.1 Hz, J = 1.7 Hz, H-7), 7.85 (1H, m, H-6), 7.91 (1H, s, -OH), and 8.19 (1H, dd, J = 8.0, J = 1.7 Hz, H-5). 13C NMR (DMSO-d6) δ: 14.3 (C-13”), 25.7 (C-2”), 29.1 (C-10”), 29.3 (C-8”), 29.4 (C-9”), 29.5 (C-7”), 29.6 (C-5”), 29.7 (C-4”), 29.8 (C-3”), 30.1 (C-11”), 31.8 (C-12”), 34.1 (C-1”), 83.0 (O-CH2-O), 111.5 (C-2”), 116.9 (C-4”), 117.3 (C-6”), 119.4 (C-8), 124.2 (C-10), 125.6 (C-6), 125.7 (C-5), 130.0 (C-5”), 132.6 (C-1”), 134.7 (C-7), 149.2 (C-3), 151.0 (C-3”), 155.5 (C-2), 155.7 (C-9), 175.1 (C-4), and 180.8 (O-C=O).

Screening tests revealed six strains of filamentous fungi capable of biotransformation of 3-methoxyflavone: a wild strain of A. niger KB, its four UV-mutants (A. niger MB, SBP, SBJ, 13/5) and the strain of P. chermesinum 113. The initial study was performed according to two procedures: in the first the substrate was added to the cultivation media at the time of inoculation with the microorganism, whereas in the second, it was added 72 hours after the inoculation. After 3, 6 and 9 days samples were analyzed for product and unreacted substrate (Table I, II). All of the tested strains of Aspergillus transformed 3-methoxyflavone into 3’-hydroxyflavon-3-yloxy methyl myristate, using either cultivation procedure. P. chermesinum 113 was also capable of this transformation, but only when the substrate was added to the cultivation media in the last phase of logarithmic growth of the mycelium (Table II). For all of the tested Aspergillus strains the biotransformation was more efficient when the substrate was added to the cultivation

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Time of incubation (days)</th>
<th>Product</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. niger KB</td>
<td>3’-hydroxyflavon-3-yloxy methyl myristate</td>
<td>53.7</td>
<td>64.8</td>
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<td></td>
<td>Unreacted substrate</td>
<td>38.2</td>
<td>24.1</td>
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<tr>
<td>A. niger MB</td>
<td>3’-hydroxyflavon-3-yloxy methyl myristate</td>
<td>25.1</td>
<td>30.7</td>
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<tr>
<td></td>
<td>Unreacted substrate</td>
<td>73.2</td>
<td>63.2</td>
</tr>
<tr>
<td>A. niger SBP</td>
<td>3’-hydroxyflavon-3-yloxy methyl myristate</td>
<td>38.7</td>
<td>50.4</td>
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<td></td>
<td>Unreacted substrate</td>
<td>60.0</td>
<td>46.3</td>
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<tr>
<td>A. niger SBJ</td>
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<td>22.5</td>
<td>23.7</td>
</tr>
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<td></td>
<td>Unreacted substrate</td>
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<td>70.9</td>
</tr>
<tr>
<td>A. niger 13/5</td>
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<td>10.1</td>
<td>20.3</td>
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<tr>
<td></td>
<td>Unreacted substrate</td>
<td>87.8</td>
<td>73.2</td>
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<table>
<thead>
<tr>
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<th>Time of incubation (days)</th>
<th>Product</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. niger KB</td>
<td>3’-hydroxyflavon-3-yloxy methyl myristate</td>
<td>13.7</td>
<td>16.2</td>
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<tr>
<td></td>
<td>Unreacted substrate</td>
<td>81.2</td>
<td>70.7</td>
</tr>
<tr>
<td>A. niger MB</td>
<td>3’-hydroxyflavon-3-yloxy methyl myristate</td>
<td>16.3</td>
<td>16.2</td>
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<tr>
<td></td>
<td>Unreacted substrate</td>
<td>80.1</td>
<td>80.0</td>
</tr>
<tr>
<td>A. niger SBP</td>
<td>3’-hydroxyflavon-3-yloxy methyl myristate</td>
<td>26.2</td>
<td>26.9</td>
</tr>
<tr>
<td></td>
<td>Unreacted substrate</td>
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<tr>
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<td>3’-hydroxyflavon-3-yloxy methyl myristate</td>
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<tr>
<td>A. niger 13/5</td>
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<td></td>
<td>Unreacted substrate</td>
<td>80.3</td>
<td>77.0</td>
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<tr>
<td>P. chermesinum 113</td>
<td>3’-hydroxyflavon-3-yloxy methyl myristate</td>
<td>16.7</td>
<td>19.2</td>
</tr>
<tr>
<td></td>
<td>Unreacted substrate</td>
<td>80.1</td>
<td>73.3</td>
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</table>
media at the time of inoculation with the microorganism. *A. niger* KB and *A. niger* SBP gave the highest yields of the product after 9 days, i.e. 69.8% and 63.1%, respectively. The least efficient strain for this biotransformation was *A. niger* 13/5 (Table I, II).

The preparative scale biotransformation of 3-methoxyflavone was carried out using the strain of *A. niger* KB in a 9-day reaction. 3'-Hydroxyflavon-3-yloxymethyl myristate was isolated in 65% yield (Scheme 1) and identified by $^1$H NMR and $^{13}$C NMR. In the $^1$H NMR spectrum the signal of the substrate methoxyl group at C-3 ($\delta = 3.81$ ppm) disappeared and a two proton singlet at $\delta = 5.22$ ppm attributed to a –O-CH$_2$-O- group appeared. Seven new signals integrating for a total of 24 protons and a singlet of 3H at $\delta = 0.86$ ppm indicate the presence of a 13-carbon alkyl chain of a saturated fatty acid (myristic acid) bonded at C-3 of the flavone by means of the ester bond. The ester carbonyl group is confirmed by the signal at $\delta = 180.8$ ppm in the $^{13}$C NMR. The presence of a hydroxyl group in the B-ring was proved by the new one-proton singlet at $\delta = 7.91$ ppm which was not present in the spectrum of the substrate. The location of the hydroxyl group was determined by analyzing the shape and chemical shift of the B-ring protons. The 2' and 6' protons and the 4' and 5' protons are nonequivalent in the $^1$H NMR of the product. In the $^{13}$C NMR the C-3' signal is shifted from $\delta = 129.2$ ppm for the substrate to $\delta = 151.0$ ppm for the product.

The mechanism of oxidative O-demethylation of methyl ethers by cytochrome P450 proposed by Watanabe is presented in Scheme 2 (Watanabe et al., 1982).

In the biotransformation of 3-methoxyflavone catalyzed by the strains of *Aspergillus* and *Penicillium* the intermediate hemiacetal is presumably esterified with myristic acid present in the cultivation mixture. Miristic acid is produced by the strains of *Aspergillus*. The presence of considerable myristic acid has been observed.
in conidiophores of *A. flavus* (Budínská et al., 1981) in the cells of *A. ochraceus* (Chavant and Sandolle, 1977) and in the cells of *A. niger* (Parang et al., 1996).

The first step of enzymatic oxidation of 3-methoxyflavone resembles metabolism of methoxy derivatives in mammals. The reaction is probably catalyzed by the fungal monooxygenases of cytochrome P-450. The proposed mechanism of the biotransformation is presented in Scheme 3. Earlier research on microbial transformations of monosubstituted flavones with methoxyl groups in the A-ring (Kostrzewa-Suslow et al., 2012) indicate that the strains of *Aspergillus* and *P. chermesinum* used for this study perform non-typical demethylation which ends with introduction of myristic acid in the favourable C-3 flavone position.

**Conclusions.** Transformation of 3-methoxyflavone in the cultures of *A. niger* (KB, MB, SBP, SBJ, 13/5) and *P. chermesinum* 113 is a two-step process involving enzymatic oxidation of both the methoxyl group and the B-ring of the flavone and then esterification of the –O-CH$_3$-OH group with myristic acid. The highest yield of transformation was achieved for the strain *A. niger* KB, when the substrate was added at the time of inoculation.

**Acknowledgements**

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**Literature**


Zeaxanthin, a C40 xanthophyll carotenoid, has potential biological applications in nutrition and human health. In this study we characterized carotenoid composition in 5 taxonomically related marine bacterial isolates from the genus *Muricauda*. The pigment was characterized using high performance liquid chromatography (HPLC) and mass spectrometry, which confirmed the presence of all-trans-zeaxanthin. *Muricauda* strains produced zeaxanthin as a predominant carotenoid. *M. flavescens* JCM 11812T produced highest yield (4.4 ± 0.2 mg L⁻¹) when cultured on marine broth at 32°C for 72 h. This is the first report on the presence of zeaxanthin among the majority of species from the genus *Muricauda*.

**Key words:** Flavobacteriaceae, *Muricauda*, marine bacteria, zeaxanthin

Zeaxanthin is a potential biomolecule having antioxidant, anticancer properties and is known to prevent age related macular degeneration (Krinsky et al., 2003). Apart from plant sources, microbes have been found as an important source of carotenoids particularly zeaxanthin (Hameed et al., 2011). Though many bacteria are known to produce zeaxanthin, the major limitation for their use in large scale commercial exploitation is that they often produce mixed carotenoids similar to plant source. Separation of zeaxanthin from total carotenoids involves multiple purification steps and turns out to be expensive. Selection of strains that grow faster and accumulate high amount of zeaxanthin can facilitate efficient extraction and purification processes. Isolation of bacteria producing biocompatible natural zeaxanthin as the major carotenoid in higher concentration will be vital for meeting large scale demand. Most important members that were reported earlier for the production of zeaxanthin are *Flavobacterium multivorum*, *Mesoralibacter zeaxanthinfaciens*, *Zeaxanthinibacter enushiensis*, *Muricauda lutoanensis* and recently described *Siansivirga zeaxanthinfaciens* (Asker et al., 2007a; b; Hameed et al., 2011; 2012). However, zeaxanthin production dynamics in closely related strains or species have not been studied.

*Muricauda* is a genus under the family Flavobacteriaceae (Bruns et al., 2001) of marine origin. It currently encompasses seven type strains known to produce characteristic orange-yellow pigmented colonies (Lee et al., 2012; Arun et al., 2009; Hwang et al., 2009; Lee et al., 2012). *M. lutoanensis* CC-HSB-11T isolated from a coastal hot-spring was first reported to produce high amounts of zeaxanthin (Hameed et al., 2011). However, no other details are available for zeaxanthin production pertaining to other species in this genus. Hence, this study was undertaken to investigate the production of zeaxanthin by other members of the genus *Muricauda*.

Four type strains of the genus *Muricauda*, namely, *M. aquimarina* JCM 11811T, *M. flavescens* JCM 11812T, *M. lutimaris* KCTC 22173T and *M. lutoanensis* KCTC 22339T and two environmental isolates, YUAB-SO-11 and YUAB-SO-45 isolated from Ullal and Cochin beaches, South-West coast of India respectively were used. All strains otherwise indicated were sub-cultured on marine agar 2216 (Difco) at 32°C, whereas, *M. lutoanensis* at 42°C and stored in 30% glycerol at –80°C. The two environmental isolates YUAB-SO-11 and YUAB-SO-45 were identified using 16S rRNA gene sequencing according to the method described earlier (Kämpfer et al., 2003). Sequence data was analyzed after multiple sequence alignment. Sequences were submitted to GenBank (GenBank Accession Number: YUAB-SO-11, JQ257008 and YUAB-SO-45, JQ346699). Optimum growth and carotenoid production of the strains was determined. Approximately 10⁶ CFU mL⁻¹
were inoculated into 100 mL flask containing 20 mL marine broth 2216 (MB) and incubated at 32°C, in the case of \textit{M. lutaoensis} at 40°C, under shaking at 150 rpm for 120 h. Cell density was determined at 24 h intervals by reading OD\textsubscript{650} using an UV-Visible spectrophotometer (Shimadzu 1800). Cell dry weight (CDW) was determined by centrifuging 1 mL of the broth culture at 7500 rpm in a pre-weighted vial and the pellet was washed twice in sterile water and dried at 80°C to constant weight. Characterization and quantification of the carotenoid was carried out as outlined by (Asker \textit{et al.}, 2007c; Hameed \textit{et al.}, 2011; Sanusi and Adebiyi, 2009). 10 mg of the lyophilized biomass was suspended in 10 mL ethanol and incubated overnight at 50°C in dark with agitation for carotenoid extraction. The carotenoid in solution was separated from the biomass by centrifugation (12000 rpm for 10 minutes at 4°C). This step was repeated until complete extraction. The solvent was evaporated under nitrogen gas in the dark and re-dissolved in 1 mL of ethanol and subjected to a full-wavelength scan (250–700 nm) using a UV-Visible spectrophotometer (UV-Vis 1800, Shimadzu, Japan). The peak obtained was plotted against calibration curve constructed using standard zeaxanthin.

Purification and identification of the carotenoid was carried out according to the methods outlined earlier using HPLC (Asker \textit{et al.}, 2007). The HPLC system with a diode array detector (L-2455, Hitachi) attached to RP column (CAPCELL PAK C18 MG S-5, 35 × 4.6 mm, 5 μm particle size) maintained at 35°C, coupled to a HPLC pump (L-2130, Hitachi) was used. Identification of the HPLC purified carotenoid was carried out using mass spectrometry consisting of a linear ion trap mass spectrometer (Thermo LTQ XL, USA) attached to a LC plus system (Thermo Scientific). Conditions (ion source, atmospheric pressure chemical ionization (APCI) source, operated in the positive ion mode; sheath gas flow (N\textsubscript{2}), 50 arbitrary units; auxiliary gas flow (N\textsubscript{2}), 10 arbitrary units; source voltage, 6 kV; capillary temperature, 300°C) were maintained. Full mass scan was selected with a separation width of 2 m/z unit for collision-induced dissociation with the collision energy of 25 eV for detection. The carotenoids were identified and confirmed using standard zeaxanthin. All the strains used in this study showed the presence of zeaxanthin exclusively.

The phylogenetic analysis of the \textit{Muricauda} type strains and two environmental isolates along with the type strains of the genus \textit{Muricauda} is given in Figure 1. The 16S rRNA gene sequence similarity in the genus \textit{Muricauda} ranged between 95.4–99.2%. Identification of the two isolates based on 16S rRNA gene sequences revealed that both the isolates YUAB-SO-11 and YUAB-SO-45 (Fig. 1) belonged to the genus \textit{Muricauda}. Strain \textit{Muricauda} sp. YUAB-SO-11 was closely related to \textit{M. aquimarina} JCM 11811\textsuperscript{T} (98.9% similarity) but could be distinguished clearly from the type strain \textit{M. aquimarina} JCM 11911\textsuperscript{T} in the phylogenetic analysis indicating that strain YUAB-SO-11 might represent a novel species in the genus \textit{Muricauda}. The strain \textit{Muricauda} sp. YUAB-SO-45 was closely related to \textit{M. olearia} (JCM 15563) (99.2% similarity). Physiological and biochemical characterization of the \textit{Muricauda} strains is provided in the Table I. Strain \textit{Muricauda} sp. YUAB-SO-11 utilized only glucose and could not utilize other carbon sources. The maximum biomass produced by different strains is listed in Table II. Among the strains, \textit{M. flavescens} JCM 11812\textsuperscript{7} showed the highest cell biomass yield (2.9 ± 0.1 g L\textsuperscript{-1}) in marine broth, whereas, strain \textit{M. lutaoensis} KCTC 22339\textsuperscript{7} showed comparatively lower biomass (1.9 ± 0.1 g L\textsuperscript{-1}) at 72 h of incubation in marine broth. Optimum growth and carotenoid production for all the strains was at pH 7–8 and 2–3% salinity (NaCl) and temperature 30–35°C except for the strain \textit{M. lutaoensis} KCTC 22339\textsuperscript{7}, which was 40°C. Preliminary spectrophotometric analysis of the ethanol-diluted crude carotenoid from all the \textit{Muricauda} strains showed typical absorption spectra with a λ\textsubscript{max} at 448 nm with shoulder peaks identical to those of the standard all-trans-zeaxanthin.
Further investigation of the pigment by HPLC analysis resolved the zeaxanthin with a prominent peak (RT, 10.8) comparable to standard zeaxanthin. This fraction was subjected to mass analysis which exhibited a parent ion at m/z 569 and collision-induced disassociation fragments of m/z 551 and 419 which were identical to that of standard all-trans-zeaxanthin (Fig. 2). The compounds corresponding to peak-2 (RT, 13.87) and peak-3 (RT, 14.65) were predicted to be other minor carotenoids respectively. The zeaxanthin produced by *M. flavescens* JCM 11812\(^{1}\) (4.4 ± 0.2 mg L\(^{-1}\)) was highest while by *M. lutimaris* KCTC 22173\(^{3}\) was the lowest (0.7 ± 0.1 mg L\(^{-1}\)) (Table II). These results indicate that zeaxanthin is the major pigment in the genus *Muricauda*.

Zeaxanthin biosynthesis occurs via the mevalonate pathway (MVA) or 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway by enzymatic reaction of β-carotene hydroxylase (*CrtZ*) which modifies the β-ring (β-carotene) to zeaxanthin (Misawa 2011). Whole genome sequencing of the strain *M. rustingensis* DSM 13258\(^{4}\) (Huntemann et al., 2012) showed the presence of *CrtZ* gene. Accumulation of high concentration of zeaxanthin in marine microorganisms including strains

### Table I

Differential physiological and biochemical characteristics of the genus *Muricauda*

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>1</th>
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<th>3</th>
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<tr>
<td></td>
<td>Optimum</td>
<td>32</td>
<td>32</td>
<td>30–37</td>
<td>30–32</td>
<td>37–45</td>
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<tr>
<td>Growth pH</td>
<td>Range</td>
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<td>5–9</td>
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<td>7–8</td>
<td>7.6</td>
<td>6.8–7.7</td>
<td>7.8</td>
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<tr>
<td>Growth with NaCl (%)</td>
<td>Range</td>
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<td>2–10</td>
<td>2–9</td>
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<td>–</td>
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<td></td>
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<td>–</td>
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<td>Hydrolysis of</td>
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<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Gelatin</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
</tbody>
</table>

Table II

Total biomass and zeaxanthin yield produced by *Muricauda* strains cultured in marine broth at 32°C or for *M. lutamensis* at 42°C at 150 rpm.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Growth time (h)</th>
<th>CDW (g/mL)</th>
<th>Zeaxanthin (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Muricauda</em> sp.YUAB-SO-11</td>
<td>48</td>
<td>2.6 ± 0.2</td>
<td>3.14 ± 0.2</td>
</tr>
<tr>
<td><em>Muricauda</em> olearia YUAB-SO-45</td>
<td>72</td>
<td>2.1 ± 0.2</td>
<td>2.16 ± 0.1</td>
</tr>
<tr>
<td><em>Muricauda</em> aquimarinia (JCM 11811(^{3}))</td>
<td>72</td>
<td>2.3 ± 0.2</td>
<td>3.5 ± 0.1</td>
</tr>
<tr>
<td><em>Muricauda</em> flavescens (JCM 11812(^{3}))</td>
<td>72</td>
<td>2.9 ± 0.1</td>
<td>4.4 ± 0.2</td>
</tr>
<tr>
<td><em>Muricauda</em> lutamensis (KCTC 22339(^{3}))</td>
<td>72</td>
<td>1.9 ± 0.1</td>
<td>3.1 ± 0.1</td>
</tr>
<tr>
<td><em>Muricauda</em> lutimaris (KCTC 22173(^{3}))</td>
<td>72</td>
<td>2.3 ± 0.1</td>
<td>0.7 ± 0.1</td>
</tr>
</tbody>
</table>
Fig. 2. LC-MS/MS analysis of the crude ethanol extract of five *Muricauda* strains (A, *Muricauda* sp. YUAB-SO-11; B, *M. olearia* YUAB-SO-45; C, *M. flavescens* JCM 11812; D, *M. aquimarina* JCM 11811; E, *M. lutimaris* KCTC 22173). Chromatogram shows a peak at 10.92 minutes compared with the zeaxanthin standard. (B) MS/MS spectrum of carotenoid from all the strains of *Muricauda* showed a parent peak at *m/z* 569, and subsequent fragments at *m/z* 551 and *m/z* 416.
from genus *Muricauda* might have an evolutionary role in survival, in membrane stabilization, UV tolerance, and as an antioxidant protecting cell from oxidative stress (Asker et al., 2012). Combining the information from this study and available published data on zeaxanthin, particularly from microbial source, it is evident that zeaxanthin production is an important phyllogenetic trait of this genus. Increasing demand for zeaxanthin, particularly from microbial source has gained significant interest due to its identical stereochemistry to natural zeaxanthin which has lead researchers to identify potential zeaxanthin producing strains. *Sphingobacterium multivorum* produced high zeaxanthin yield (Bhosale et al., 2004), whereas *Muricauda* species produce zeaxanthin as a single predominant carotenoid in higher quantities indicating strains from this genus likely be potential candidates for large scale production of zeaxanthin. Comparison of carotenoid distribution in *Muricauda* strains with their 16S rRNA gene phylogeny indicates that the carotenoid distribution and composition is highly conserved between the species. In the genus *Muricauda*, the uniform distribution of zeaxanthin strongly suggests that the carotenoid biosynthetic pathway may be less evolutionary elastic resulting in only certain structural types existing without giving further scope for the diversification of carotenoids within the genus (Klassen 2009; 2010).

The result of this study further enhances our current limited knowledge regarding the distribution and diversity of carotenoid in family *Flavobacteriaceae*. There is further scope for investigating the evolution of carotenoids in these species within the concept of biotechnology and exploiting their metabolic diversity.

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**Literature**


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