

Gram-negative Microorganisms Isolated from Fish and their Sensitivity against Essential Oils

Miroslava Kačániová^{1,2}, Alina Klūga³, Margarita Terentjeva³, Simona Kunová¹,
Czesław Puchalski²

¹Slovak University of Agriculture, Faculty of Biotechnology and Food Sciences, Nitra 949 76, Tr. A. Hlinku 2, Slovakia

²University of Rzeszow, Faculty of Biology and Agriculture, 35-601 Rzeszow, Zelwerowicza St. 4, Poland

³Latvia University of Life Sciences and Technologies, Faculty of Veterinary Medicine, Institute of Food and Environmental Hygiene, Jelgava, LV-3004, K. Helmaņa iela 8, Latvia

Abstract

Essential oils may inhibit Gram-negative microbiota, a causative agent of spoilage of fresh fish. The aim of this study was to detect antibacterial activity of essential oils against Gram-negative bacteria isolated from fish. The antimicrobial activity of *Citrus paradisi* peel oil, *Citrus reticulata* peel oil, *Juniperus communis* fruit oil, *Eucalyptus globulus* leaf oil and *Cananga odorata* flower oil was studied. The microorganisms tested were *Pseudomonas fluorescens*, *P. frederiksbergensis*, *P. gessardii*, *P. ludensis*, *P. proteolitica*, *Shewanella baltica*, *Yersinia ruckeri* and *Y. enterocolitica*. Essential oils were tested with disc diffusion method and method of minimal inhibition concentration. The best antimicrobial activity of *Cananga odorata* flower peel oil and *Juniperus communis* fruit oil was found against *Pseudomonas* spp. and less against *Shewanella baltica*, *Yersinia ruckeri* and *Y. enterocolitica*. *Citrus paradisi* peel oil and *Citrus reticulata* peel oil were most active against *Yersinia* spp. *Eucalyptus globulus* leaf oil showed good antimicrobial effect against *Shewanella baltica*.

Keywords: fish microbiota, essential oils, antibiotic resistance

1. Introduction

Round goby (*Neogobius melanostomus*) was indirectly introduced from the Black and Caspian Sea to Europe and northern Europe. First observation of round goby in the Baltic Sea was in Gdansk in Poland in 1991. In Latvia, the round goby was described near to Liepāja in 2004. Nowadays, the round goby is widespread alongside the Latvian Baltic Sea coastline but not in freshwaters [1–7].

The marine environment has been one of the best maintained over time. However, environmental problems derived from aquaculture, such as overfishing, introduction of new species with

different microflora brought from other countries, the excess of biomass and waste generated, as well as the indiscriminate use of antimicrobial compounds began to produce a major change in the microflora of both marine and inland waters. In example, pathogenic microorganisms such as *Pseudomonas*, both the terrestrial and aquatic organisms, begin to be dangerous unexplored [8]. Strains of genus *Pseudomonas* are curved rods, Gram-negative, motile with one or more polar flagellum, catalase and oxidase-positive, strictly aerobic but some strains can use nitrate in anaerobic conditions. The genus *Shewanella* comprises species which are widely distributed in aquatic environments. *Shewanella* species have been isolated from marine fish [9,10], freshwater fish [11] and mollusks [12]. Some *Shewanella* species such as *S. marisflavi* [13] and *S. alga* [14] and freshwater as *S.*

* Corresponding author: Miroslava Kačániová,
+421376414494, miroslava.kacaniova@gmail.com

putrefaciens have been described as the pathogens of marine organisms. Some strains of these species could provide an antimicrobial effect against certain fish pathogens and improve the tolerance of stress induced by high density fish farming [15]. Furthermore, *Shewanella putrefaciens*, is not only a member of the microbial association found in fish from temperate waters, but they also contribute significantly to the spoilage of fish stored under different conditions. *Yersinia* is a genus of Gram-negative, rod-shaped, facultative anaerobes within the family *Yersiniaceae*. *Yersinia* represents several pathogenic species, which cause diseases in humans and animals, including fish. *Yersinia ruckeri* is a causative agent of enteric redmouth disease in various species of salmonids worldwide. It was described in rainbow trout in the Hagerman Valley of Idaho, USA in the 1950s [16]. *Yersinia enterocolitica*, Gram-negative rod, belongs to *Enterobacteriaceae* family, has been recognized as an important food and water-borne pathogen [17-19]. Any edible raw animal food (fish, meat, pork, and poultry) may carry *Y. enterocolitica* and cause infection in humans. During the last two decades, *Y. enterocolitica* was isolated from animals, vegetables, various environments, water and human all over the world [20].

Pseudomonas aeruginosa, *Staphylococcus aureus*, *Salmonella* spp, coagulase-negative *Staphylococcus*, *Shigella*, *Enterococcus* sp. and *Escherichia coli* are amongst some of the main bacteria with multidrug resistance and are included in the category of community and hospital acquired pathogens. This has resulted in the strong demand of new antibiotics by consumers against pathogens [21] and an interest has been developed by the scientific community for using herbal medicines with antimicrobial properties. Plants and other natural sources can provide a huge range of complex and structurally diverse compounds. Plant extracts and essential oils possess antifungal, antibacterial, and antiviral properties and have been screened on a global scale as potential sources of novel antimicrobial compounds, agents promoting food preservation, and alternatives to treat infectious diseases [22,23].

The aim of this study was to detect antibacterial activity of essential oils against Gram-negative bacteria isolated from fish.

2. Materials and methods

Microorganisms

Eight strains of microorganisms were tested in this study, including Gram-negative bacteria *Pseudomonas fluorescens*, *P. frederiksbergensis*, *P. gessardii*, *P. ludensis*, *P. proteolitica*, *Shewanella baltica*, *Yersinia ruckeri* and *Y. enterocolitica*. All tested strains were collected from the fish *Neogobius melanostomus* and were identified with MALDI TOF MS Biotyper with score >2.20. The bacterial suspensions were cultured in the Muller Hinton broth (MHB, Oxoid, Basingstoke, United Kingdom) at 37 °C for 24 h before inoculation.

Antibiotic susceptibility testing

All isolated bacterial strains were tested for antimicrobial susceptibility. An amount of 100µl of bacterial suspension in 0.9 % NaCl with 0.5 McF° density was spread with sterile L-rods onto Mueller Hinton agar (Oxoid, UK). Antimicrobial susceptibility was detected with disc diffusion method and following antibiotics discs (Oxoid, UK) were used: doripenem DRP (10µg), imipenem IMP (10µg), ciprofloxacin CPR (10µg), levofloxacin LVX (10µg), amikacin AMK (10µg), gentamicin GEN (10µg). Inoculated agars were incubated at 35±2°C for 16-20 h according to the EUCAST [24]. Interpretation of inhibition zones were done in line with EUCAST [25].

Essential oils

For antimicrobial activity, the essential oils *Citrus paradisi* peel oil, *Citrus reticulata* peel oil, *Juniperus communis* fruit oil, *Eucalyptus globulus* leaf oil, and *Cananga odorata* flower oil were studied.

Detection of susceptibility of bacteria to essential oils

For disc diffusion method, a suspension of the tested microorganism (0.1 ml of 10⁵ cfu/ml) was used in experiment. Suspension was transferred onto Mueller Hinton Agar (MHA, Oxoid, Basingstoke, United Kingdom). Filter paper discs (6 mm in diameter) were impregnated with 15 µl of oil and placed onto inoculated agars, which were kept at 4 °C for 2 h, and then at 37°C for 24 h. The diameters of the inhibition zones were measured in millimeters. All the tests were performed in triplicate.

The minimum inhibitory concentration (MIC) for detection of antimicrobial activity of essential oils

was tested as was described in Kačániová et al. [26] study.

3. Results and discussion

Our results showed that identified bacterial strains from fish gut were resistant to antibiotics but wide

variation between the bacterial species were observed. *Yersinia enterocolitica* was the most resistant strain isolated from fish was (Table 1).

Our results showed resistance to more than one class of antibiotic. Multiple drug resistance has been reported in a number of studies of fish pathogens and aquaculture environments [27].

Table 1 Antibiotic susceptibility of bacteria isolated from fish gut

Bacterial strains	Antibiotics 10 µg					
	DRP	IMP	CPR	LVX	AMK	GEN
<i>Pseudomonas fluorescens</i>	R	R	S	S	S	S
<i>P. frederiksbergensis</i>	S	R	S	S	S	S
<i>P. gessardii</i>	R	S	R	S	R	S
<i>P. ludensis</i>	S	S	S	R	S	S
<i>P. proteolitica</i>	S	R	S	S	S	R
<i>Shewanella baltica</i>	S	S	S	S	S	S
<i>Yersinia ruckeri</i>	R	R	R	S	R	S
<i>Y. enterocolitica</i>	R	R	R	S	S	R

Legend to antimicrobial drug abbreviation: doripenem DRP (10µg), imipenem IMP (10µg), ciprofloxacin CPR (10µg), levofloxacin LVX (10µg), amikacin AMK (10µg), gentamicin GEN (10µg)

Table 2 Antimicrobial activity of essential oils detected with disc diffusion method in mm

Bacterial strains	Tested essential oil				
	<i>Citrus paradisi</i>	<i>Citrus reticulata</i>	<i>Juniperus communis</i>	<i>Eucalyptus globulus</i>	<i>Cananga odorata</i>
<i>Pseudomonas fluorescens</i>	7.33±1.15	7.66±0.58	13.33±1.53	5.33±0.58	14.33±1.15
<i>P. frederiksbergensis</i>	6.67±1.53	7.33±0.58	13.00±1.00	5.67±0.58	14.67±1.53
<i>P. gessardii</i>	6.33±1.15	7.67±0.58	14.67±2.52	6.00±1.00	15.33±1.53
<i>P. ludensis</i>	7.33±0.58	6.67±1.53	13.67±2.31	5.67±1.15	14.67±0.58
<i>P. proteolitica</i>	7.67±0.58	6.33±1.15	14.66±0.58	5.33±1.53	15.33±1.52
<i>Shewanella baltica</i>	4.67±0.58	4.67±0.58	6.33±0.58	16.00±1.73	5.00±1.00
<i>Yersinia ruckeri</i>	11.67±0.58	10.33±1.53	4.67±0.58	4.33±0.58	5.33±0.58
<i>Y. enterocolitica</i>	11.33±1.15	10.67±2.89	5.33±1.15	5.33±0.58	5.33±0.58

Table 2 Antimicrobial activity expressed as minimum inhibitory concentration (MIC50) against bacteria species of EOs

Bacterial strains	Tested essential oil				
	<i>Citrus paradisi</i>	<i>Citrus reticulata</i>	<i>Juniperus communis</i>	<i>Eucalyptus globulus</i>	<i>Cananga odorata</i>
<i>Pseudomonas fluorescens</i>	50.00	50.00	6.25	50.00	12.50
<i>P. frederiksbergensis</i>	25.00	25.00	6.25	100.00	12.50
<i>P. gessardii</i>	25.00	50.00	12.50	50.00	12.50
<i>P. ludensis</i>	50.00	50.00	12.50	50.00	6.25
<i>P. proteolitica</i>	50.00	25.00	12.50	50.00	12.50
<i>Shewanella baltica</i>	100.00	50.00	25.00	6.25	100.00
<i>Yersinia ruckeri</i>	6.25	12.50	25.00	25.00	50.00
<i>Y. enterocolitica</i>	6.25	12.50	25.00	25.00	50.00

Antimicrobial activity of essential oils against bacteria isolated from fish are shown in Table 2. The most effective was *Eucalyptus globulus* essential oil tested with disc diffusion method against *Shewanella baltica* (16.00 ± 1.73).

The antibacterial activity of eucalyptus EO on fish spoilage and nine fish-borne pathogenic bacteria was described in Debbarma et al., 2013 study [28]. The results indicated that the eucalyptus leaves EO showed different degrees of growth inhibition and *B. subtilis* was the most inhibited bacteria, followed by *L. monocytogenes* and *S. aureus* [28]. The essential oils of *Cannaga odorata* flower peel oil and *Juniperuss communis* fruit oil showed the best antimicrobial activity against *Pseudomonas gessardii* and *P. proteolitica* while the EO of *Citrus paradisi* peel oil and *Citrus reticulata* peel oil were most effective against *Yersinia ruckeri* and *Y. enterocolitica*.

Although the uses of ylang-ylang oil and its safety as food ingredient have been reviewed previously, the studies on the pharmacological activities of the *Cananga odorata* plant were still very limited. A very brief review on the antibacterial, antifungal, amebicidal, and cytotoxic activities of the ylang-ylang EOs was published [29]. The synergistic effects of ylang-ylang with different combinations of EOs for treatment of microbial infections have been reported. The combinations of ylang-ylang oil and thyme oil were significantly more effective against *S. aureus* ATCC 25923 and its synergistic effect was observed between both of the essential oils in which the inhibition zone was increased by 38.4% as compared to thyme oil alone. An antagonism of ylang-ylang oil applied with thyme oil against *Escherichia coli* ATCC 25922 was observed with a reduction of the inhibition zone by 48.9% when compared to thyme oil alone [30]. Similarly, the blended EOs of lavender, clary sage and ylang-ylang oils in the ratio 3 : 4 : 3 displayed a strong antibacterial and antifungal activities against *Staphylococcus aureus* ATCC 6538, *Staphylococcus epidermidis*, *Escherichia coli* ATCC 25923, *Pseudomonas aeruginosa* ATCC 9027 and *Candida albicans* ATCC 10231 [31].

Minimal inhibitory concentrations of the EO against bacteria isolated from fish are shown in Table 4. Minimal inhibitory concentration of the EOs showed the similar results as were identified with disc diffusion method. The best antimicrobial activity of *Cannaga odorata* flower peel oil

and *Juniperuss communis* fruit oil on *Pseudomonas* ssp. was revealed.

EO of *Citrus* inhibited growth of *Escherichia coli*, *Staphylococcus aureus*, *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *diacetylactis*, *Leuconostoc mesenteroides* subsp. *dextranicum* and *Lactobacillus plantarum* in Vasek et al., 2015 study [32].

Javed et al., 2011 [33] concluded that among the various Pakistani *Citrus* species, the EO of *Citrus reticulata* var. Mandarin was highly effective against a wide range of food spoiling bacteria. The strongest antimicrobial activity of *J. communis* EO against *Salmonella enterica* subsp. *enterica* was observed with the EO obtained at the 0–3 DT. The EO of *J. communis* obtained at the 0–3, 3–5, and 80–160 min DTs showed greater antimicrobial activity against *Klebsiella pneumonia*, compared with the EO obtained from the 160 to 240 DT. The strongest antimicrobial activity of *J. communis* EO against *Staphylococcus aureus* subsp. *aureus* and *Candida glabrata* was observed with EO from the 160 to 240 DT. For *J. communis*, the effect of DT was not significant on *C. perfringens*, *Candida albicans*, and *Candida tropicalis*, and their overall means were 14.5, 13.5, and 13.2 respectively.

4. Conclusions

The *Cannaga odorata* flower peel oil and *Juniperuss communis* fruit EOs have shown antibacterial activity at low concentration against fish spoilage bacteria within the genus *Pseudomonas*, whereas, the EO of *Eucalyptus globulus* has exhibited antibacterial activity only at higher concentration with the expectation *Shewanella*. Antibacterial activity of EOs against antibiotic resistant *Y. enterocolitica* demonstrates the potential of antibacterial activity of EOs.

The antibacterial activity demonstrated by EOs against the 8 selected fish spoilage and fish-borne pathogenic bacteria is an indication of the antibacterial potential of *Citrus paradisi* peel oil, *Citrus reticulata* peel oil, *Juniperus communis* fruit oil, *Eucalyptus globulus* leaf oil and *Cananga odorata* flower oil as a source of antibacterial substances for the development and formulation of antibiotics and preservatives with broad spectrum of activity. The safety and quality of fish and fishery products may be improved with

an application of the EOs. Further investigations are required to evaluate the antibacterial efficacy of EOs in real food systems based on fish and fishery products.

Acknowledgements

The study was supported by the European Community project No 26220220180: Building Research Centre „AgroBioTech“ and by grant of Slovak Research and Development Agency No. VEGA 1/0411/17.

References

1. Kotta, J., Nurksa, K., Puntila, R., Ojaveer, H., Shipping and natural environmental conditions determine the distribution of the invasive non-indigenous round goby *Neogobius melanostomus* in a regional sea. *Estuarine, Coastal and Shelf Science*, 2016, 169, 15-24
2. Sapota, M.R., Online Database of the European Network on Invasive Alien Species – NOBANIS 2012. Available: www.nobanis.org
3. Ojaveer, H., Galil, B.S., Lehtiniemi, M., Christoffersen, M., Clink, S., Florin, A.B., Gruszka, P., Puntila, R., Behrens, J.W., Twenty five years of invasion: management of the round goby *Neogobius melanostomus* in the Baltic Sea, *Management of Biological Invasions*, 2015, 6(4), 329-339
4. Bolonina, A., Comoglio, C., Calles, O., Kunickis, M., Strategies for mitigating the impact of hydropower plants on the stocks of diadromous species in the Daugava river, *Energy Procedia*. 2016, 95, 81-88
5. Vakulenko, I., Myroshnychenko, I., Approaches to the organization of the energy efficient activity at the regional level in the context of limited budget resources during the transformation of energy market paradigm, *Environmental and climate technologies*, 2015, 1, 59-76
6. Froese, R., Pauly, D. FishBase 2016. Available: <http://fishbase.org/>
7. Barisa, A., Dzene, I., Rosa, M., Dobraja, K., Waste-to-biomethane Concept Application: A Case Study of Valmiera City in Latvia, *Environmental and climate technologies*, 2015, 1, 49-58
8. Avendaño-Herrera, R., Núñez, S., Barja, J.L., Toranzo, A.E., Evolution of drug resistance and minimum inhibitory concentration to enrofloxacin in *Tenacibaculum maritimum* strains isolated in fish farms, *Aquaculture International*, 2008, 16, 1-11.
9. Satomi, M., Vogel, B.F., Gram, L., Venkateswaran, K., *Shewanella putrefaciens* sp nov and *Shewanella morhuae* sp nov, isolated from marine fish of the Baltic Sea, *International Journal of Systematic Evolutionary Microbiology*, 2006, 56, 243-249
10. Satomi, M., Vogel, B.F., Venkateswaran, K., Gram, L., Description of *Shewanella glacialis* sp nov and *Shewanella algidipiscicola* sp nov., isolated from marine fish of the Danish Baltic Sea, and proposal that *Shewanella affinis* is a later heterotypic synonym of *Shewanella colwelliana*, *International Journal of Systematic Evolutionary Microbiology*, 2007, 57, 347-352
11. Goldschmidt-Clermont, E., Wahli, T., Frey, J., Burr, S.E., Identification of bacteria from the normal flora of perch, *Perca fluviatilis* L., and evaluation of their inhibitory potential towards *Aeromonas* species, *Journal of Fish Disease*, 2008, 31, 353-359
12. Richards, G.P., Watson, M.A., Crane III, E.J., Burt, I.G., Bushek, D., *Shewanella* and *Photobacterium* spp. in oysters and seawater from the Delaware bay, *Applied Environmental Microbiology*, 2008, 74, 3323-3327
13. Li, H., Qiao, G., Li, Q.W., Zhou, K.M., Won, D.H., Xu, S.I. Park, Biological characteristics and pathogenicity of a highly pathogenic *Shewanella marisflavi* infecting sea cucumber, *Apostichopus japonicus*, *Journal of Fish Disease*, 2010, 33, 865-877
14. Beleneva, I.A., Kухlevskii, A.D., Characterization of *Vibrio gigantis* and *Vibrio pomeroyi* isolated from invertebrates of Peter the Great Bay, Sea of Japan, *Microbiology*, 2009, 79, 402-407
15. Varela, J.L., Ruíz-Jarabo, I., Vargas-Chacoff, L., Arijo, S., León Rubio, J.M., García Millán, I., Martín del Río, M.P., Moriñigo, M.A., Mancera, J.M., Dietary administration of probiotic Pdp11 promotes growth and improves stress tolerance to high stocking density in gilthead sea bream *Sparus auratus*, *Aquaculture*, 2010, 265-271
16. Ross, A.J., Rucker, R.R., Ewing, W.H., Description of a bacterium associated with redmouth disease of rainbow trout (*Salmo gairdneri*), *Canadian Journal of Microbiology* 1966, 12, 763-770
17. Eden, K.V., Rosenberg, M.L., Stoopler, M., Wood, B., Highsmith, A.K., Skaliy, P., Wells, J., Feeley, J.C., Waterborne gastroenteritis at a ski resort associated with the isolation of *Yersinia enterocolitica*, *Public Health Reports*, 1977, 92, 245-250
18. Keet, E.E., *Yersinia enterocolitica* septicemia. *New York State Journal of Medicine*, 1974, 74, 2226-2230
19. Bottone, E.J., *Yersinia enterocolitica*: The charisma continues, *Clinical Microbiology Review*, 1997, 10, 257-276
20. Hellmann, E., Heinrich, G., Growth rates of two virulence plasmids carrying *Yersinia enterocolitica* after contamination of heated milk, raw minced pork and vegetables, *Zentralblatt für Bakteriologie Mikrobiologie und Hygiene B*, 1985, 82, 1-16
21. Fisher, K., Phillips, C., Potential antimicrobial uses of essential oils in food: Is citrus the answer? *Trends Food Sciences Technology*, 2008, 19, 156-164

22. Safaei-Ghomi, J., Ahd, A.A.. Antimicrobial and antifungal properties of the essential oil and methanol extracts of *Eucalyptus largiflorens* and *Eucalyptus intertexta*, Pharmacognosy Magazine, 2010, 6, 172-175
23. Astani, A., Reichling, J., Schnitzler, P., Comparative study on the antiviral activity of selected monoterpenes derived from essential oils. Phototherapy Research, 2010, 24, 673-679
24. European Committee on Antimicrobial Susceptibility Testing (EUCAST). Antimicrobial susceptibility testing: Eucast disk diffusion method, version 3.0 from April 2013
25. European Committee on Antimicrobial Susceptibility Testing (EUCAST). Breakpoint tables for interpretation of MICs and zone diameters, version 5.0 valid from 2015-01-01
26. Kačániová, M., Terentjeva, M., Vukovic, N., Puchalski, C., Roychoudhury, S., Kunová, S., Kluga A., Tokár, M., Kluz M., Ivanišová E., The antioxidant and antimicrobial activity of essential oils against *Pseudomonas* spp. isolated from fish, Saudi Pharmaceutical Journal, 2017, 25(8), 1108-1116
27. Hatha, M., Vivekanandam, A.A., Joice, G.J., Christol, G.J., Antibiotic resistance pattern of motile aeromonads from farm raised freshwater fish, International Journal of Food Microbiology, 2005, 98, 131-134
28. Debbarma, J., Kkishore, P., Nnayak, B.B., Kannuchamy, N., Gudipati, V.T., Antibacterial activity of ginger, eucalyptus and sweet orange peel essential oils on fish-borne bacteria, Journal of Food Processing and Preservation, 2013, 37, 1022-103
29. Burdock, G.A., Carabin, I.G., Safety assessment of Ylang-Ylang (*Cananga* spp.) as a food ingredient, Food and Chemical Toxicology, 2008, 46(2), 433-445
30. Kon, K., Rai, M., Antibacterial activity of *Thymus vulgaris* essential oil alone and in combination with other essential oils, Nusantara Bioscience, 2012, 4(2), 50-56
31. Tadtong, S., Suppawat, S., Tintawee, A., Saramas, P., Jareonvong, S., Hongratanaworakit, T., Antimicrobial activity of blended essential oil preparation, Natural Product Communications, 2012, 7(10), 1401-1404
32. Vasek, O.M., Cáceres, L.M., Chamorro, E.R., Velasco, G.A., Antibacterial activity of *Citrus paradise* essential oil, Journal of Natural Products, 2015, 8, 16-26
33. Javed, S., Javaid, A., Mahmood, Z., Javaid, A., Nasim, F., Biocidal activity of citrus peel essential oils against some food spoilage bacteria, Journal of Medicinal Plants Research, 2011, 5(16), 3697-3701
34. Zheljzakov, V.D., Semerdjieva, I.V., Dincheva, I., Kačániová, M., Astatkief, T., Radoukovag, T., Schlegel, V., Antimicrobial and antioxidant activity of *Juniper galbuli* essential oil constituents eluted at different times, Industrial Crops and Products, 2017, 109, 529-537.