Survival of *Arcobacter butzleri* and *Arcobacter cryaerophilus* strains in the presence of sea buckthorn extracts

SIMONA KUČEROVÁ – DAVID ŠILHA – JARMILA VYTŘASOVÁ – BLANKA ŠVECOVÁ

Summary

Bacteria of the genus *Arcobacter* can be a cause of serious diseases not only for humans but also for animals. Therefore, their presence in food has to be eliminated. The strains of *Arcobacter butzleri* and *A. cryaerophilus* were used in this experiment as isolates, obtained from foodstuffs, and also as collection strains. The aim of this study was to test the survival of arcobacters in the presence of sea buckthorn (*Hippophae rhamnoides* L.) extracts (ethanolic extract from sea buckthorn, and extract from sea buckthorn in phosphate buffered saline). Antimicrobial sensitivity was tested by agar diffusion well test, followed by determination of minimum inhibitory concentration for individual extracts. To verify the inhibitory effect of the extracts, testing the antimicrobial effect of artificially inoculated foodstuff was carried out. Extracts were also analysed using high-performance liquid chromatography with diode-array detector, with the aim to approximately identify their substances. The results of this study show that sea buckthorn extracts could be suitable natural antimicrobial substances active, in particular against *A. butzleri* and *A. cryaerophilus*.

Keywords

Arcobacter; antimicrobial sensitivity testing; natural antimicrobial substances; sea buckthorn; high-performance liquid chromatography

Bacteria of the genus *Arcobacter*, formerly known as "aerotolerant campylobacters", belonging to the family *Campylobacteraceae* [1], have many common characteristics with the genus *Campylobacter*. Arcobacters are Gram-negative, spiral-shaped rods that can grow under microaerobic conditions [2] at temperatures between 15 °C and 30 °C. This is in contrast to the temperature at which campylobacters grow [1].

Genus *Arcobacter* currently contains 22 species [3–5]. Some of these can cause abortion, mastitis and gastrointestinal diseases in animals [6], and also bacteremia, endocarditis, peritonitis and diarrhea in humans [7]. Solid evidence is available of the connection of human diseases and some species of arcobacters, namely, *A. butzleri, A. cryaerophilus*, *A. skirrowii* and *A. thereius* [8, 9].

Despite the large progress in medical technologies and medical findings, infectious diseases are still the most common cause of illness and deaths in the world [10]. Currently, the spread of infectious diseases caused by multiresistant strains of bacteria is a big issue [11]. Previous studies on arcobacters showed that they are resistant to many types of antibiotics, hence more effective antimicrobials should be developed [12, 13].

In the past, natural products have been a priceless source of therapeutic possibilities [14]. Natural products are still considered as a main source of new healing resources, with the possibility of effectively targeting a large number of different diseases [15]. Antimicrobial substances obtained from plants are the most common secondary metabolites. These metabolites are mostly phenols or their substituted derivatives. They provide different benfits, including antimicrobial characteristics [16]. Apart from these compounds, quinones, flavonoids, coumarins, terpenes and alkaloids possess some antimicrobial activity [17].

Sea buckthorn (*Hippophae rhamnoides* L.) is

Simona Kučerová, David Šilha, Jarmila Vytřasová, Department of Biological and Biochemical Sciences, Faculty of Chemical Technology, University of Pardubice, Studentská 573, 532 10 Pardubice, Czech Republic.
Blanka Švecová, Department of Analytical Chemistry, Faculty of Chemical Technology, University of Pardubice, Studentská 573, 532 10 Pardubice, Czech Republic.

Correspondence author:
David Šilha, e-mail: David.Silha@upce.cz

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a leafy bush with a distinctive resistance against dryness, cold and saltiness of the ground. Yellow to yellow-orange edible berries of sea buckthorn contain a wide variety of nutrients and bioactive compounds, including acyl lipids, carotenoids, tocopherols, sterols, phenolic compounds, vitamins, sugars, sugar alcohols, glucose derivatives, inositol, free amino acids and minerals [18]. *O*-Ethyl-β-D-glucopyranoside is a typical compound found in sea buckthorn berries, but it is not commonly found in other fruits [19].

The aim of this project was to investigate the survival of arcobacters in the presence of sea buckthorn extracts, in the case of collection strains and also strains isolated from foodstuffs. For this study, two most common species of arcobacters found in the Czech Republic were selected [20]. Further, an analysis of prepared extracts, aimed at approximate identification of the spectrum of substances, was performed.

MATERIALS AND METHODS

Bacterial cultures and growth

The strain of *Arcobacter butzleri* CCUG 30484 from the culture collection (University of Göteborg, Göteborg, Sweden) was used for testing, together with *A. cryaerophilus* CCM 3934 obtained from Czech Collection of Microorganisms (Masaryk University, Brno, Czech Republic). Further strains used were *A. butzleri* UPa 2015/6 (isolate from the turkey neck) and *A. cryaerophilus* UPa 2015/16 (isolate from salmon) from the collection of microorganisms (University of Pardubice, Pardubice, Czech Republic). Both latter strains were isolated from foodstuffs for this study.

Microbial cultures were grown on tryptone soya agar (TSA, HiMedia, Mumbai, India) at 30 °C for 48 h and then stored at 4 °C. The strains were subcultured on TSA at 30 °C for 48 h before the experiment. After the cultivation on TSA agar, a suspension of cells in the physiological saline solution was prepared (McFarland turbidity from 3×10^8 CFU·ml^{-1} to 9×10^8 CFU·ml^{-1}). Suspension of cells was diluted to a density of approximately 10^7 CFU·ml^{-1} and was used further for testing. For the verification of the exact number of cells in the prepared suspension, the presumptive density of 10^3 CFU·ml^{-1} was counted on Mueller-Hinton agar (HiMedia).

Isolation of *Arcobacter* spp. from foodstuffs

For the purpose of the isolation of *Arcobacter* spp. from foodstuffs, 25 g of a foodstuff sample was thoroughly homogenized in 225 ml of *Arcobacter* broth with cefoperazone, amphotericin B, teicoplanin selective (CAT) supplement (Oxoid, Basingstoke, United Kingdom) and incubated at 30 °C for 48 h. Further, passive filtration of 100 μl of the culture, through a membrane filter with pore diameter of 0.45 μm (Pall, Port Washington, New York, USA), was done. Passive filtration of the bacterial microflora proceeded for 30 min at 25 °C and then the filter was removed from the agar medium. The incubation at 30 °C lasted 48 h. Suspect colonies of *Arcobacter* spp. were picked and placed on TSA agar and, after incubation, were identified by multiplex polymerase chain reaction [21, 22].

Extracts from sea buckthorn

Sea buckthorn used for preparation of the extracts came from a small farmer in Pardubice (Czech Republic), where berries were freshly harvested. A sample (20 g) was washed with distilled water, cut into smaller pieces and immersed in 100 ml of 96% ethanol (Lach-Ner, Neratovice, Czech Republic). Extracts were held in a closed jar, in the dark, at 25 °C for 48 h, and were stirred occasionally. Suspensions were filtered through a qualitative filter paper, Grade 1 (Whatman, Maidstone, United Kingdom) and put into the thermostat set on 37 °C. After the complete evaporation of ethanol, 15 ml of two different solvents (96% ethanol and phosphate buffered saline, PBS) were used to dissolve the dry extract. Each obtained extract was first sterilized by filtration through a membrane filter with the pore diameter of 0.45 μm, collected in a sterile plastic test tube and then kept in the dark with a temperature of 4 °C for a maximum of 6 months. The final concentration of both extracts was approximately 60 mg·ml^{-1}.

Determination of antimicrobial effect of sea buckthorn extracts

The agar diffusion well test was used for antimicrobial activity testing. The suspension of bacterial cells (2 ml) with a density of 10^7 CFU·ml^{-1} was inoculated on Mueller-Hinton agar and kept to soak at 25 °C for 15 min. After this time, the excessive cell suspension was removed. Holes were made in the inoculated agar plate (diameter 11 mm). The tested extract was pipetted into holes and the plate was incubated at 30 °C for 48 h. The level of the extract in the holes was checked regularly during the cultivation, and extract was added when needed. After the cultivation, inhibition zones were measured. The experiment was carried out three times repeatedly and the results were expressed as average ± standard deviation.

Determination of minimum inhibitory concentration

Minimum inhibitory concentration (*MIC*) was determined by a microdilution method in 96-well microtiter plates (Nunclon Delta Surface; Nunc, Roskilde, Denmark). Into the wells, 50 μl of Mueller-Hinton broth was pipetted, followed by 50 μl of the microbial suspension with a cell density of 10^7 CFU·ml^{-1}. Further 50 μl of the diluted extract was added in each well, so the final concentration in wells was 0.6–7.3 mg·ml^{-1}. After cultivation at 30 °C for 48 h, the content of each well was inoculated on Mueller-Hinton agar. *MIC* was stated as the lowest concentration of the extract that inhibited the growth of bacteria. This experiment was carried out three times repeatedly and the results are expressed as average ± standard deviation.

Determination of the antimicrobial effect in a model of a real foodstuff

Commercial baby food containing meat and vegetables (Hamé, Kunovice, Czech Republic) was used. A amount of 10 g of sterile baby food, 100 μl bacterial suspension of *A. butzleri* CCUG 30484 with the cell density of 10^7 CFU·ml^{-1}, 4 ml of ethanolic extract from sea buckthorn, and 40 ml physiological saline solution were put together and all were carefully homogenized. This mixture was diluted 10× in physiological saline solution and 100 μl of this mixture was immediately inoculated on Mueller-Hinton agar (time t_0). The prepared mixture was incubated at 4 °C and, after 48 h, another inoculation on Mueller-Hinton agar (time t_1) was carried out.

At the same time, the experiment with a pure solvent (addition of 4 ml 96% ethanol) and a blind test (without addition of the extract) was carried out. After the cultivation, the growth on plates was evaluated and the effect of the extract on arcobacters in the real foodstuff was assessed. This experiment was carried out two times repeatedly and the results are graphically demonstrated as a logarithm of the number of colony forming units per millilitre, in accordance with time, ± standard deviation. In case that a high number of colonies was on plates, dilution in physiological saline was performed and then the results were re-calculated.

HPLC analysis

Prior to the chromatographic analysis, the prepared extracts were diluted in a ratio of 1 : 10. The extract in PBS was diluted with water, and the ethanolic extract was diluted with ethanol. The diluted extracts were filtered through a 0.45 μm (pore size) polytetrafluoroethylene filter (Merck, Darmstadt, Germany) and these extracts were ready for HPLC analysis.

Analyses were performed using the high-performance liquid chromatography (HPLC) system: an Agilent 1290 Infinity liquid chromatograph (Agilent, Palo Alto, California, USA) equipped with a degasser, a pump, an autosampler, a diode-array UV detector and a thermostatted column compartment. Chromatographic conditions were as follows: column Ascentis Express C18 (150 mm × 3 mm, particle size 2.7 μm; Sigma-Aldrich, St. Louis, Missouri, USA), mobile phases (MP) water acidified with acetic acid (MP-A; approx. pH 3) and acetonitrile (MP-B). In both cases, the flow rate was 0.5 ml·min^{-1}, and the gradient elution was as follows: 0 min 0% MP-B, 5 min 10% MP-B, 15 min 40% MP-B, 25 min 100% MP-B. The sample volume was 5 μl and separation temperature of 35 °C. For spectrophotometric detection, a wavelength of 280 nm was found to be optimal. Eluted substances were iden-

Tab. 1. Inhibitory effects of extracts from sea buckthorn on selected arcobacters.

	A. butzleri CCUG 30484	*A. butzleri* UPa 2015/6	*A. cryaerophilus* CCM 3934	*A. cryaerophilus* UPa 2015/16
	Average size of inhibition zone [mm]			
Ethanolic extract	28.5 ± 2.4	24.5 ± 2.1	23.0 ± 1.0	25.5 ± 0.7
Extract in PBS	18.0 ± 2.0	20.5 ± 0.7	28.0 ± 0.0	20.3 ± 1.5
Ethanol (96%)	16.0 ± 0.1	15.3 ± 0.1	15.3 ± 0.1	15.5 ± 0.1
PBS	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	MIC [mg·ml^{-1}]			
Ethanolic extract	1.8 ± 0.0	1.8 ± 0.0	1.2 ± 0.0	0.9 ± 0.4
Extract in PBS	1.8 ± 0.0	1.8 ± 0.0	1.8 ± 0.0	1.8 ± 0.0

Results are expressed as average ± standard deviation (n = 3).
MIC – minimum inhibitory concentration; PBS – phosphate buffered saline.

tified according to their retention times (R_t) and comparison with literature [23]. Following standards were used, which important for two groups of substances occur in the extract: rutin (≥ 95%, Sigma-Aldrich), quercetin (≥ 90%, Fluka, Buchs, Switzerland), apigenin (≥ 95%, Sigma-Aldrich) and kaempferol (≥ 97%, Sigma-Aldrich).

RESULTS AND DISCUSSION

Determination of antimicrobial effect of sea buckthorn extracts

Because the resistance to antibiotics has an increasing trend, it is necessary to study the antimicrobial activity of natural materials and extracts prepared therefrom, particularly against pathogenic bacteria. In this study, extracts prepared from fresh sea buckthorn berries were tested against the pathogenic bacteria of the genus *Arcobacter*. In recent years, these bacteria are often found not only in foodstuffs, but also in water [8, 24, 25]. The sensitivity of arcobacters to some external influences has been described in previous studies. For example, it was found that arcobacters are sensitive to extracts from cinnamon, bearberry, chamomile, sage and rosemary [26], and barely survive on various metallic materials used in the food industry [27].

In this study, extracts of sea buckthorn were prepared in ethanol and PBS buffer. Both extracts were tested as potential antimicrobial agents against arcobacters. The average sizes of the inhibition zones for all tested strains are presented in Tab. 1. Comparison of data on the activity of the two tested extracts is detailed in Fig. 1. Both prepared extracts showed some antimicrobial activity against bacteria of the genus *Arcobacter*, but their effectiveness differed between individual strains. The most antimicrobially effective against the collection strain *A. butzleri* CCUG 30484 was the ethanolic extract. The recorded size of the inhibition zone was 28.5 mm ± 2.4 mm. Antimicrobial efficacy of the ethanolic extract from sea buckthorn against *A. butzleri* UPa 2015/6 (isolated from turkey neck) was lower, the inhibition zone being 24.5 mm ± 2.1 mm. The results for the species *A. cryaerophilus* suggest that, in this case, the strain isolated from food was more sensitive to the inhibitory effect of the ethanolic extract from the sea buckthorn. For the collection strain *A. cryaerophilus* CCM 3934, an inhibitory zone size of 23.0 mm ± 1.0 mm was recorded. This was in contrast to the strain isolated from salmon (UPa 2015/16), for which the recorded size of the inhibition zone was 25.5 mm ± 0.7 mm. For the extract prepared in ethanol, a predictable antimicrobial effect of the solvent alone was observed. Nevertheless, even in the case of sea buckthorn extract prepared in PBS buffer, interesting values of size of inhibition zones (18.0 mm to 28.0 mm) were found. The results are reported in Tab. 1, suggesting interesting inhibitory effects of the extracts against selected arcobacters. From these preliminary results, it is not clear whether collection strains are more sensitive than isolates from foodstuffs. For clarification, a larger set of strains would have to be tested in the future.

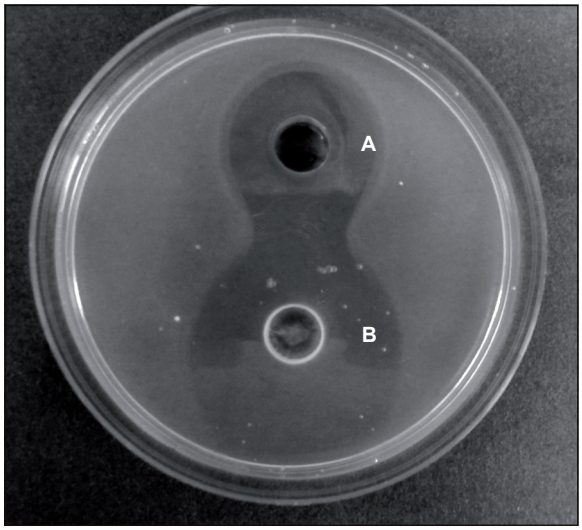

Fig. 1. Inhibition zones of sea buckthorn extracts with *A. butzleri* CCUG 30484, after 48 h at 30 °C.

A – extract in phosphate buffered saline, B – ethanolic extract.

The antimicrobial effect of water and ethanol sea buckthorn leaf extracts was studied previously with other Gram-negative and Gram-positive bacteria [28]. The aqueous extract from sea buckthorn leaves, was the strongest inhibitory effects against *Pseudomonas aeruginosa* (average size of inhibition zones 18 mm), and ethanolic extract inhibited the most *Bacillus cereus* (average size of inhibition zones 19 mm) [28].

Another study investigated the influence of water and ethanol infusions of sea buckthorn on seven strains of bacteria. The aqueous and ethanolic infusions inhibited *Bacillus subtilis* and *Kocuria rhizophila*. Only the ethanolic infusion suppressed growth of *Escherichia coli* and *Lactobacillus acidophillus*. Both infusions were not effective against *Listeria monocytogenes*, *Bifidobacterium bifidum* and *Campylobacter jejuni* [29]. Ineffectiveness of sea buckthorn extract against *Campylobacter jejuni* is unexpected, in view of results of this study, because of its common charac-

teristics with genus *Arcobacter*. However, differences between different strains were reported also in that study. Different preparation of extracts also certainly played a great role.

Determination of minimum inhibitory concentration

MIC values of sea buckthorn extracts was determined by the microdilution method, the results are presented in Tab. 1. The results show that the lowest *MIC* values were determined in the case of strains of *A. cryaerophilus, A. cryaerophilus* CCM 3934 showed a *MIC* for the ethanolic extract from the sea buckthorn of 1.2 mg·ml^{-1}. The strain *A. cryaerophilus* UPa 2015/16 showed a *MIC* of 0.9 mg·ml^{-1} ± 0.4 mg·ml^{-1}. For the other tested arcobacters, *MIC* was 1.8 mg·ml^{-1}. *MIC* of the extract from sea buckthorn in PBS was the same for all tested strains, 1.8 mg·ml^{-1}.

Effects of sea buckthorn seed extracts on growth of different bacteria was monitored in a previous study. Chloroform, acetone and methanolic extracts of seeds were tested against bacteria of the genus *Bacillus*, *Listeria monocytogenes* and *Yersinia enterocolitica*. Methanolic extract was found to be the most effective, followed by acetone and chloroform extracts. *Y. enterocolitica* was the most resistant to all the extracts, and higher *MIC* values were obtained for it (approx. 0.35–0.75 mg·ml^{-1}). The lowest *MIC* values were obtained for *Bacillus cereus*. Higher resistance of Gram-negative bacteria to external agents has been earlier reported, being attributed to the presence of lipopolysaccharides in their outer membranes, which makes them inherently more resistant [30].

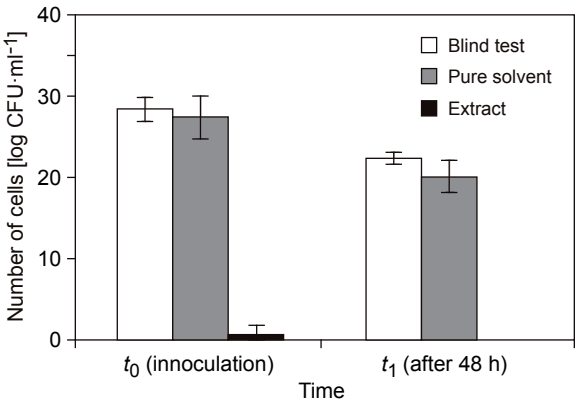

Fig. 2. Survival of *A. butzleri* CCUG 30484 in a model foodstuff with ethanolic extract from the sea buckthorn.

The results are expressed as average ± standard deviation (n = 2).

Determination of the antimicrobial effect in a model of real foodstuffs

Ethanolic extract of sea buckthorn was further tested to determine antimicrobial effects in a model of real foodstuffs. This parameter was studied because there are different conditions for the survival of microorganisms, in comparison with common laboratory testing. For this experiment, the strain *A. butzleri* CCUG 30484 was selected, and testing was performed in an artificially inoculated foodstuff. For this purpose, a meat-vegetable baby food was chosen. Bacterial counts after 48 h are presented in Fig. 2. Survival of arcobacters, in the mixture containing the sample of food with the ethanolic extract from the sea buckthorn (t_0), was very low, (0.75 ± 1.3) log CFU·ml^{-1}. This probably means that there was a significant inhibition of viable cells due to the presence of the ethanolic extract from sea buckthorn. Surviving arcobacters in the control mixture (blind test), and a mixture of the sample of food with 96% ethanol, was approximately at the same level, (28.5 ± 1.5) log CFU·ml^{-1} and (27.5 ± 2.7) log CFU·ml^{-1}, respectively. It can be stated that the inoculated cells of *A. butzleri* CCUG 30484 were not inhibited by the presence of the solvent alone (96% ethanol).

Further experiments were carried out in the same way, but the detection of arcobacter survival was monitored after 48 h. after the prepared mixture was kept at 4 °C (t_1). A lower number of cells was detected than in the previous case (comparing with the case where the inoculating onto agar was performed immediately after mixing of all the parts, at t_0). A lower number of cells could have been caused by pH 4.35 of the meat-vegetable baby food, which is a much lower pH level than that optimal for growth of bacteria of the genus *Arcobacter*. Arcobacters can grow at pH 5.5–5.9, but most strains grow between pH 6.8–8.0 [31]. After a relatively long exposure (48 h), the elimination might have been caused by the sample itself. In the mixture containing the sample of food with the addition of ethanolic extract from sea buckthorn (t_1), no *Arcobacter* cells grew (Fig. 2). This indicates that after a 48 h exposure to ethanolic sea buckthorn extract, all present arcobacters were inhibited. Surviving arcobacters in the control mixture (blind test) and a mixture of the sample of food with 96% ethanol were approximately at the same level, (22.5 ± 0.8) log CFU·ml^{-1} and (20.2 ± 2.0)log CFU·ml^{-1}, respectively.

HPLC analysis

Comparison of Fig. 3A and Fig. 3B shows clearly that rutinosides (R_t of rutin is 8.3 min) are easier to be extracted into PBS than into etha-

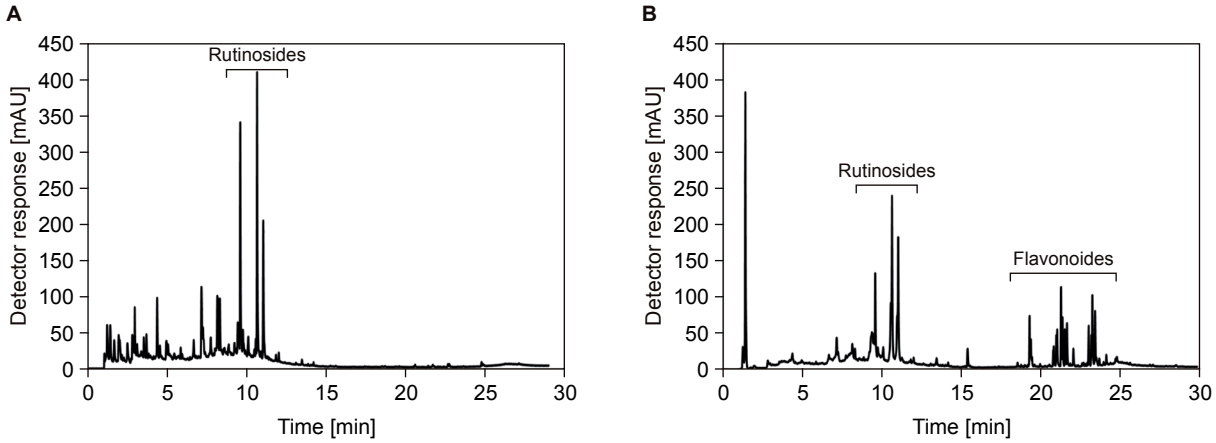

Fig. 3. HPLC analysis of sea buckthorn extracts.

A – extract in phosphate buffered saline, B – ethanolic extract.

nol. Peaks at the beginning of the chromatogram (Fig. 3A) belong to polar polyphenolic substances, which are easier to extract into a more polar solvent, such as PBS. In contrast, other flavonoids (R_t of quercetin is 21.5 min, R_t of apigenin is 22.1 min, R_t of kaempferol is 23.9 min), which are less polar than rutinosides, were extracted only into ethanol and not into PBS. Rutinosides are very common glycoside compounds derived from rutin, and belong to the group of polyphenolic compounds, which have several biological activities, including the antimicrobial activity. The antimicrobial effect of rutin was already proven against bacteria from the genus *Staphylococcus* and against the Gram-negative *Moraxella catarrhalis* [32]. Due to the fact that both types of extract inhibited arcobacters, we can presume that rutinosides have an antimicrobial effect against bacteria of the genus *Arcobacter*.

CONCLUSION

The number of multiresistant strains of bacteria has been increasing rapidly in the last few years. The same can be said for arcobacters, where the resistance against some common antibiotics has been proven. It is well known that natural products are a source of antimicrobial substances, and above all, go with today´s trend of using natural products. The tested sea buckthorn extracts had an inhibitory effect on strains of bacteria of the genus *Arcobacter.* If other tests extend these findings, sea buckthorn may have a future of an antimicrobial agent against bacteria of the genus *Arcobacter*.

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