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Influence of natural substances on the survival of *Arcobacter*-like species and their biofilm formation

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Abstract

Alimentary and infectious diseases are one of the leading causes of death worldwide. A related issue is the overuse of chemicals, disinfectants, drugs (especially antibiotics), which increases the development of bacterial resistance. Bacterial diseases caused by multi-drug resistant strains of bacteria are a serious problem. Bacteria acquire resistance by various mechanisms, e.g. mutations, uptake of new genetic information or growth in biofilms.

Antimicrobial efficacy of natural compounds could help with the growing problem of antimicrobial and antibiofilm resistance. Many plant matrices show some antimicrobial effects. In this study, biofilm formation was evaluated in strains isolated from food, water and clinical samples belonging to the *Campylobacteraceae* and *Arcobacteraceae* families. Biofilm formation was monitored on different surfaces using the modified Christensen method in an aerobic or microaerophilic atmosphere after incubation 24 or 72 h. Furthermore, the effects of several substances with potential antimicrobial activity (antibiotics, hydrosols and oil extracts) on planctonic cells and cells in the biofilm structure of *Arcobacter*-like species and other selected strains were monitored. The oil extracts were analyzed by liquid chromatography and the obtained hydrosols by gas chromatography.

Keywords

Arcobacter-like species, antibioitics, antimicrobial substances, biofilm, *Campylobacter* spp., extracts, hydrosols, natural matrices

Abstrakt

Alimentární a infekční onemocnění jsou jednou z nejčastějších příčin úmrtí na celém světě. Problematikou, která se s tímto tématem pojí, je nadměrné užívání chemických látek, dezinfekčních prostředků, léčiv (zejména antibiotik), což zvyšuje tvorbu bakteriální rezistence. Bakteriální onemocnění způsobená multirezistentními kmeny bakterií jsou závažným problémem. Bakterie získávají rezistenci různými mechanismy, např. mutacemi, přijímáním nových genetických informací nebo růstem v biofilmu.

Antimikrobiální účinnost přírodních látek by mohla pomoci s rostoucím problémem antimikrobiální a antibiofilmové rezistence. Řada rostlinných matric vykazuje určité antimikrobiální účinky. V rámci studie byla hodnocena biofilmová tvorba u kmenů izolovaných z potravin, vody a klinických vzorků patřících do čeledi *Campylobacteraceae* a *Arcobacteraceae*. Tvorba biofilmu byla monitorována v aerobní nebo mikroaerofilní atmosféře po 24 nebo 72 hod. kultivace na různém kultivačním povrchu, a to pomocí modifikované Christensenovy metody. Dále byly sledovány účinky několika látek s potenciálním antimikrobiálním účinkem (antibiotika, hydrosoly a extrakty z olejů) na planktonní buňky a buňky ve struktuře biofilmu *Arcobacter*-like species a dalších vybraných kmenů. Extrakty z olejů byly analyzovány pomocí kapalinové chromatografie a získané hydrosoly pomocí plynové chromatografie.

Klíčová slova

Arcobacter-like species, antibiotika, antimikrobiální látky, biofilm, *Campylobacter* spp., extrakty, hydrosoly, přírodní matrice

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1 Introduction

1.1 *Arcobacter-like species*

The first mention of these microorganisms dates back to 1977, as they were previously mistaken for campylobacters or later aerotolerant campylobacters. In 1991 that arcobacters were described in more detail (Vandamme et al., 1991) as a separate genus *Arcobacter*. However, these bacteria are currently placed in a separate family, *Arcobacteraceae* (Waite et al., 2018). It has been proposed that the original genus *Arcobacter* be divided into six different taxonomic units, namely the genera *Arcobacter* (*A.*), *Aliarcobacter* (*Al.*), *Pseudarcobacter* (*Ps.*), *Haloarcobacter* (*H.*), *Malacobacter* (*M.*) and *Poseidonibacter* (*Po.*) (Pérez-Cataluna et al., 2019). The genus *Arcomarinus* (*Ar.*) has also been proposed (Chieffi et al., 2020). Currently, a total of about 36 species are classified as *Arcobacter*-like species (Chieffi et al., 2020). *Arcobacter*-like species do not differ much in morphology and metabolic characteristics from campylobacters. However, they can grow under aerobic and microaerophilic conditions at 15–37 °C, this is a characteristic feature that differentiates *Arcobacter*-like species from the genus *Campylobacter*. Some strains are capable of biofilm formation (Kjeldgaard et al., 2009). There are no internationally accepted criteria for antibiotic susceptibility testing for *Arcobacter*-like species, the criteria according to CLSI (Clinical and Laboratory Standards Institute) or EUCAST (European Commite on Antimicrobial Susceptibility Testing) recommended for *Campylobacter* or *Enterobacteriaceae* family are mainly used (Šilha et al., 2017). Antibiotics recommended for the treatment of gastrointestinal arcobacterioses include aminoglycosides and tetracyclines (Rathlavath et al., 2017).

1.2 *Selected substances with antimicrobial potential*

Nowadays the natural antimicrobials and their extracts are a highly sought-after alternative to synthetic preservatives (Atanasov et al., 2015). The main function of all antimicrobial compounds is to suppress the viability of microorganisms. The field of antimicrobial research focuses on the appropriate use or synergistic efficacy with other antimicrobials. However, the use of antimicrobials should not contribute to the development of resistance in microorganisms. The development of effective combinations of natural antimicrobials could lead to new methods of disinfection or reduction of the viability of microorganisms, not only their plantonic but also biofilm forms (Brenes et al., 2007).

1.3 *Bacterial biofilm*

The first picture of a biofilm was published in 1977 and showed bacteria adhering to each other with a certain layer of matrix. Today, it is even estimated that bacterial biofilms are responsible for approximately 80 % of all bacterial infections (Paluch et al., 2020). A microbial biofilm is defined as a community of microorganisms surrounded by their own matrix. Microorganisms forming biofilms can adhere to a variety of surfaces and can be found virtually anywhere. They are very often found on medical implants such as catheters, joint replacements, cardiac implants or contact lenses. The ability of microorganisms to form biofilms is associated with their natural resistance

to the effects of antibiotics. The combination of antibiofilm compounds with effective antibiotics is necessary for the effective elimination of bacterial biofilms (Vasudevan, 2014). Synergistic application of antibiotics is relatively effective, but the doses of antibiotics after which the biofilm matrix is no longer recovered are too high for in vivo administration. It can be concluded that the future of biofilm diseases and their subsequent treatment is uncertain (Martelli et al., 2018).

2 Aim of PhD study

The aim of the research in this dissertation was to deepen the knowledge of *Arcobacter*-like species. These are bacteria that have been described for a relatively long time, but many of their properties have not been described yet. The focus of the study can be divided into the following thematic sections.

- Biofilm formation of *Arcobacter*-like species under different experimental conditions and on different material surfaces.
- Evaluation of the antimicrobial effect of selected antibiotics on planctonic cells as well as their influence on bacterial biofilm formation.
- Evaluation of the biological effect of hydrosols obtained by distillation of natural matrices, both on planctonic cells and biofilm formation. Chemical analysis of the prepared hydrosols.
- Evaluation of the biological effect of olive oil extracts on planctonic cells and biofilm formation. Chemical analysis of the prepared oil extracts.

3 Experimental part

3.1 *Microbial strains and cell suspension preparation*

A total of 64 *Arcobacter*-like species, 10 strains of the genus *Campylobacter* and 5 other microbial strains were used in the experiments (Table 1 and 2). The individual strains from the Collection of Microorganisms of the University of Gothenburg, Sweden (labelled CCUG), from the Collection of Microorganisms of the University of Ghent, Belgium (labelled LMG), from the Czech Collection of Microorganisms of Masaryk University in Brno (labelled CCM), from the internal collection of microorganisms of the University of Pardubice (labelled UPa), from the Hospital of the Pardubice Region and. s. - Pardubice Hospital (labelled NP), from the Hospital of the Pardubice Region a.s. - Svitavská nemocnice (labelled NS) and from the Regional Hospital Náchod, a.s. (labelled ONN) were used. Cultivation of *Arcobacter*-like species was carried out at 30 °C for 48–72 h. Cultivation of *Campylobacter* species was carried out at 42 °C for 48–72 h. Cultivation of *E. coli*, *S. aureus*, *P. aeruginosa*, *E. faecalis* was carried out at 37 °C for 24 h, and in the case of *C. albicans*, cultivation was carried out at 37 °C for 48 h. For the experiments, a cell suspension with cell densities corresponding to 1.5–3×10^8 CFU/ml was prepared. The cell density was always adjusted by dilution to the desired concentration for the experimental purpose.

Table 1 – *Arcobacter*-like species used in the study.

***Arcobacter*-like species**		
Al. butzleri CCUG 30848	*Al. butzleri* UPa 2015/10[B]	*Al. cryaerophilus* CCM 3934
Al. butzleri LMG 10828	*Al. butzleri* UPa 2015/11[B]	*Al. cryaerophilus* CCM 7050
Al. butzleri UPa 2012/3[A]	*Al. butzleri* UPa 2015/12[B]	*Al. cryaerophilus* UPa 2012/1[A]
Al. butzleri UPa 2013/3[B]	*Al. butzleri* UPa 2015/13[B]	*Al. cryaerophilus* UPa 2013/1[B]
Al. butzleri UPa 2013/6	*Al. butzleri* UPa 2015/14[B]	*Al. cryaerophilus* UPa 2013/12[A]
Al. butzleri UPa 2013/9	*Al. butzleri* UPa 2013/3[B]	*Al. cryaerophilus* UPa 2013/13[A]
Al. butzleri UPa 2013/10[A]	*Al. butzleri* UPa 2015/15[B]	*Al. cryaerophilus* UPa 2013/14[A]
Al. butzleri UPa 2013/15[A]	*Al. butzleri* UPa 2015/16[B]	*Al. cryaerophilus* UPa 2013/16[A]
Al. butzleri UPa 2013/30[B]	*Al. butzleri* UPa 2015/18[B]	*Al. cryaerophilus* UPa 2013/17[B]
Al. butzleri UPa 2013/31[B]	*Al. butzleri* UPa 2015/19[B]	*Al. cryaerophilus* UPa 2013/28[B]
Al. butzleri UPa 2013/32[B]	*Al. butzleri* UPa 2015/20[C]	*Al. cryaerophilus* UPa 2013/35[A]
Al. butzleri UPa 2013/33[B]	*Al. butzleri* UPa 2015/25[B]	*Al. cryaerophilus* UPa 2014/58[B]
Al. butzleri UPa 2013/36[A]	*Al. butzleri* UPa 24A[B]	*Al. cryaerophilus* UPa 2014/58A[B]
Al. butzleri UPa 2013/36[A]	*Al. butzleri* UPa 30B[B]	*Al. cryaerophilus* UPa 2014/58D[B]
Al. butzleri UPa 2013/37[B]	*Al. butzleri* UPa 39-3[C]	*Al. cryaerophilus* UPa 2014/59[B]
Al. butzleri UPa 2014/51[B]	*Al. butzleri* UPa 49B[B]	*Al. cryaerophilus* UPa 130[B]
Al. butzleri UPa 2014/54[B]	*Al. butzleri* UPa 65A[B]	*Pd. defluvii* LMG 29654
Al. butzleri UPa 2015/1[B]	*Al. butzleri* UPa 132A[B]	*Al. lanthieri* LMG 28517
Al. butzleri UPa 2015/5[A]	*Al. butzleri* UPa 138A[B]	*Al. skirrowii* LMG 6621
Al. butzleri UPa 2015/6[B]	*Al. butzleri* UPa 141B[B]	*Al. thereius* LMG 24488
Al. butzleri UPa 2015/7[B]	*Al. butzleri* UPa KK[B]	
Al. butzleri UPa 2015/9[B]	*Al. cryaerophilus* CCM 3933	

Legend: *Al.* - *Aliarcobacter*; Pd. - *Pseudarcobacter*; [A] - strains isolated from water; [B] - strains isolated from food, [C] - clinical isolates; UPa - strains from the internal collection of the University of Pardubice; CCUG - strains obtained from the collection of the University of Göteborg, LMG - strains obtained from the Belgian collection Co-ordinated Collections of Microorganisms, CCM - strains from the Czech Collection of Microorganisms.

Table 2 – *Campylobacter* strains and other strains used in the study.

***Campylobacter* strains**	**Other strains**
C. jejuni CCM 6214	*S. aureus* CCM 4232
C. jejuni NP 2896^{C}	*E. faecalis* CCM 4224
C. jejuni NS 3668^{C}	*P. aeruginosa* CCM 3955
C. jejuni NS 3800^{C}	*E. coli* CCM 3954
C. jejuni NS 4088^{C}	*C. albicans* CCM 8186
C. jejuni NS 4091^{C}	
C. coli NP 2395^{C}	
C. coli NS 3803^{C}	
C. coli NS 4062^{C}	
Campylobacter sp. ONN 366^{C}	

Legend: *C.* - *Campylobacter*; *S. aureus* - *Staphyloccocus aureus*; *E. faecalis* - *Entereoccocus faecalis*; *P. aeruginosa* - *Pseudomonas aeruginosa*; *E. coli* - *Escherichia coli*; *C. albicans* - *Candida albicans*; ^{C} - clinical isolates; CCM - strains from Czech Collection of Microorganisms; NP - strains from Pardubice Hospital; NS - strains from Svitavy Hospital; ONN - strains from Regional Hospital Náchod.

3.2 *Monitoring of biofilm formation on glass and plastic surface*

Biofilm formation was monitored in borosilicate glass tubes into which BHI broth (2 ml; Himedia, India) and cell suspension (100 µl) were placed. Biofilm formation was monitored after incubation under specific conditions (30 °C for *Arcobacter*-like species and 42 °C for *Campylobacter* spp.) for 24 h and 72 h, respectively, under aerobic or microaerophilic atmosphere. After incubation, the contents of the tubes were poured into the waste and the tubes were washed with sterile distilled water and dried at 37 °C. Subsequently, the inside of the glass tubes was stained with 1% crystal violet for 15 min. The tubes were then washed again with distilled water and dried completely at 37 °C. Evaluation of biofilm formation was performed visually by comparison with the weakly adherent strain *E. coli* CCM 3954 and the strongly adherent strain *S. aureus* CCM 4224. All experiments were repeated at least three times. Non-biofilm forming strains were labelled (N), biofilm forming strains were categorised as weakly adherent (W), moderately adherent (M) and strongly adherent (S).

For biofilm determination on plastic surface, method in 96-well microtiter flat-bottomed polystyrene plates (SPE, Korea) were used in which BHI broth (90 µl) and cell suspension (10 µl) were pipetted at a density corresponding to 1.5×10^{8} CFU/ml. *Arcobacter*-like species were cultured at 30 °C for 24 h or 72 h in aerobic or microaerophilic condition. Bacteria of the genus *Campylobacter* were cultured at 42 °C for 24 h or 72 h, again in either aerobic or microaerophilic conditions. The microtiter plate was spectrophotometrically measured at 595 nm before and after incubation. Subsequently, the contents of the entire plate were shaken to waste, the plate was carefully washed with distilled water and dried at 37 °C. After drying, 100 µl of 2% sodium acetate was pipetted into the plate (15 min) to fix the biofilm formed. The contents of the plate were shaken to waste, and the plate thus treated was stained with 1% crystal violet (100 µl) for 15 min. The stained plate was washed with distilled water and dried at 37 °C. The formed biofilm-crystal violet complex was solubilized with 96% ethanol (200 µl), and then the absorbance in the new plate was measured

at 595 nm. Strains were categorized according to a classification system as non-adherent (N; OD ≤ ODC), weakly adherent (W; ODC < OD ≤ 2×ODC), moderately adherent (M; 2×ODC < OD ≤ 4×ODC) and strongly adherent (S; 4×ODC < OD), where the ODC (cut-off OD) value is defined as three times the standard deviation above the mean OD of the negative control (blank value). The measured and calculated OD/ODC values (0.111/0.120) were the same for all measurements.

3.3 *Effect of antibiotics on the survival of Arcobacter-like species*

3.3.1 *Determination of the minimum inhibitory (bactericidal) concentration*

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined by the microdilution method in 96-well flat-bottomed polystyrene microtiter plates. Stock solutions of ATBs were prepared from their concentration series (0.03–256 mg/l) in M-H II broth (MH-II, Merck, Germany). Each well (total volume 100 µl) was inoculated with cell suspension, and the final cell density corresponded to 1.5×10^6 CFU/ml. Each well was inoculated onto non-selective TSA medium after incubation for 24 h at 30 °C under aerobic conditions. The MIC value was evaluated as the lowest concentration of antibiotic that inhibited visible growth of microorganisms (99.9 %). In addition, MIC_{50} and MIC_{90} values were also expressed as the concentrations at which 50 % and 90 % of the tested strains were visibly inhibited. The minimum bactericidal concentration (MBC) was defined as the lowest antibiotic concentration required to kill 99.9 % of the bacteria subcultured on TSA medium. Each experiment was performed at least four times.

As there are no internationally accepted criteria for testing the susceptibility of *Arcobacter*-like species to antibiotics, strains were classified based on the breakpoint values recommended for *Campylobacter coli* (erythromycin, ciprofloxacin and tetracycline) and the *Enterobacteriaceae* family according to CLSI and EUCAST (Švarcová et al., 2022).

3.3.2 *Monitoring of biofilm formation in the presence of antibiotics*

The effect of antibiotics on biofilm formation was studied in 96-well flat-bottomed polystyrene microtiter plates. The microtiter plate contained a positive control for biofilm formation (PK) and a concentration series of antibiotics to which a cell suspension of 1.5×10^8 CFU/ml was added. Determination of biofilm formation was carried out using an identical procedure, see Section 2.3. Six antibiotics from Sigma-Aldrich (Sigma-Aldrich, USA), namely, ampicillin (AMP), ciprofloxacin (CIP), clindamycin (DA), erythromycin (E), gentamicin (GE) and tetracycline (TE), were selected for the experimental work. Stock solutions of the antibiotics were prepared in distilled water at a concentration of 1024 mg/l. Prior to use, the antibiotic stock solutions were stored according to the manufacturer's instructions at 2–8 °C and -20 °C, respectively.

3.4 *Chemical composition and biological activity of hydrosols*

Four plant matrices were chosen for the preparation of the hydrosols, petals of *Lavandula angustifolia* Mill. originating from Croatia (Mediate, Czech Republic), leaves of *Laurus nobilis* L. originating in Brazil (Kasia Vera, Czech Republic), cloves (*Syzygium aromaticum* L.) originating in Madagascar (Kasia Vera, Czech Republic) and fennel seeds (*Foeniculum vulgare* Mill.) originating in the Czech Republic (Kasia Vera, Czech Republic). Hydrosols of these matrices were obtained by hydrodistillation (HD) and steam distillation (SD). The obtained hydrosols (SD and HD) were further concentrated on a Separon SGX C18 column (modified octadecylsilica gel, 60 μm, 0.5 g) using 96% ethanol. The sorbent in the column was first activated with methanol (5 ml) and finally washed with sterile distilled water (10 ml). A sample of hydrosol (100 ml) was forced through the column and then the concentrated hydrosol was eluted from the column using ethanol (2 ml). In this way, 50× concentrated hydrosols of H_{SD_SPE} and H_{HD_SPE} were obtained. The concentrated hydrosols were analyzed by gas chromatography with flame ionization detector (GC-FID) and by gas chromatography with mass spectrometry (GC-MS).

Chemical analysis of hydrosols by GC-MS

The analysis of the obtained hydrosols was carried out using a GC-2010 gas chromatograph coupled with a GC-MS-QP2010 Plus mass selective detector (both Shimadzu, Japan) and a Combi Pal Autosampler (CTC Analytics, Switzerland). The chromatograph was equipped with an SLB-5ms capillary column (30 m × 0.25 mm, film thickness 2.5 μm; Supelco, USA). Helium 5.0 (Linde, Czech Republic) was used as carrier gas at a constant flow rate of 30 cm/s. The temperature program was set at 40 ºC for 3 min, then the thermostat was heated to 250 ºC at a rate of 2 ºC/min and the final temperature was maintained for 10 min. The injection and transducer to detector temperature was set at 200 °C. 1 μl of hydrosol was dispensed at a split ratio of 1:50. The mass spectrometer was operated in electron ionization mode, the ionization energy was 70 eV and ions in the range of m/z 33-500 were measured. Identification of essential oil constituents was performed using mass spectra that were compared with the National Institute of Standards and Technology's (NIST '14 Mass Spectral Library) and the Flavour & Fragrance Natural & Synthetic Compounds GC-MS library), and verification was performed by comparison of retention indices (Adams, 2007; Linstrom and Mallard, 2001).

Chemical analysis of hydrosols by GC-FID

The analysis of the obtained hydrosols was carried out using a GC 2010 gas chromatograph in conjunction with a flame ionization detector (Shimadzu, Japan) and a Combi Pal autosampler (CTC Analytics, Switzerland). The chromatographic conditions were identical to those of the GC-MS analyses. The injection temperature was set at 200 ºC and the detector temperature at 260 ºC. The sample volume (1 μl) was injected at a split ratio of 1:50. The identification of the compounds was done based on the calculated retention indices, using the retention index according to Van den Dool

and Kratz and the results of compound identification from MS analysis. The measured retention times of the sample components, the retention times of a series of alkanes (C8-C40) and the known retention indices according to van den Dool and Kratz were compared. The percentages of each compound in the samples were calculated from the ratios of the areas of the respective peaks and the total area of the peaks in the chromatogram.

3.4.1 *Evaluation of the antimicrobial efficacy of hydrosols*

Cell suspensions with cell densities corresponding to $1.5–3\times10^8$ CFU/ml were plated onto the appropriate agar medium using a swab. Then, blank discs impregnated with 8 µl of the tested hydrosol (or ethanol as control) were applied using a diosensor. After culture (30–37 °C), inhibition zones were read using a BACMED 6iG2 automatic analyzer (Aspiag, Czech Republic). Each experiment was performed at least three times and the displayed inhibition zones are expressed as mean value including standard deviation. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined by the microdilution method in 96-well flat-bottomed polystyrene microtiter plates. Concentration series of antibiotics (0.02–50 %) in M-H II broth were prepared from stock hydrosol solutions. Each well (total volume of 100 µl) was inoculated with cell suspension, the final cell density corresponded to 1.5×10^6 CFU/ml. After culturing for 24 h at 30 °C under aerobic conditions, inoculation was performed on M-H agar in the case of *Arcobacter*-like species. The MIC value was evaluated as the lowest concentration of antibiotic that inhibited visible growth of microorganisms (99.9 %). The minimum bactericidal concentration (MBC) was defined as the lowest antibiotic concentration required to kill 99.9 % of microorganisms subcultured on M-H agar or Sabouraud dextrose agar. Each experiment was performed at least four times.

3.4.2 *Evaluation of biofilm formation in the presence of hydrosols*

The effect of hydrosols on biofilm formation was investigated in 96-well flat-bottomed polystyrene microtiter plates. The microtiter plate contained a positive control for biofilm formation (PK) and a concentration range of antibiotics to which a cell suspension of 1.5×10^8 CFU/ml was added. Determination of biofilm formation was performed using an identical procedure, see Section 2.3.

3.5 *Chemical composition and biological activity of oil extracts*

Five samples of olive oil of different quality were purchased from distributors in the Czech Republic. Oil extract samples were prepared by extraction with PBS buffer (BEOO) and also with distilled water (WEOO), always by mixing the oil sample with the extraction reagent in a 1:1 ratio. The emulsion thus formed was extensively extracted on a shaker to extract the substance into the aqueous phase. This was followed by centrifugation (9000 rpm) for 5 min, after which the olive oil extract (WEOO and BEOO) was collected to serve as a matrix for subsequent testing.

Chemical analysis of oil extracts by HPLC-CoulArray

The HPLC system with electrochemical detection included a CoulArray detector (ESA, USA) with CoulArray application software for Windows, Vacuum Degasser DG 3014 (ECOM, Czech Republic), chromatography pumps type LC-10AD, column thermostat, mixer, pulse damper and metering valve (10 µl). A Gemini C18 chromatography column (150×3 mm, 3 µm) (all ESA, USA) was chosen. A 5 mM aqueous solution of ammonium acetate with formic acid (pH~3) was selected as mobile phase A (MF A) and acetonitrile as mobile phase B (MF B). The mobile phase flow rate was set at 0.4 ml/min with a linear gradient of elution (0 min, 0–30 min: 5–60 % MP B). The column temperature was set at 40 °C. Potentials of 200-900 mV were applied to each channel. The total phenolic content of the extracts was determined using the Folin-Ciocalteu reagent method. External calibration was performed using gallic acid in the concentration range of 0.1–50 mg/l. To 1 ml of extract was added 1 ml of 95% ethanol, 5 ml of distilled water and 0.5 ml of Folin-Ciocalteu reagent. After 5 min, 1 ml of 5% sodium carbonate solution was added. This mixture was left for 60 min at laboratory temperature. The absorbance of the mixture was then measured using a Genesys 50 UV-VIS spectrophotometer (Sigma-Aldrich, USA) at a wavelength of 765 nm. The results were expressed as gallic acid equivalents (GAE). All determinations were performed in quadruplicate and the results are expressed as mean including standard deviation.

3.5.1 *Evaluation of the antimicrobial efficacy of oil extracts*

To test the antimicrobial effects of the oil extracts, a cell suspension with a density of 3×10^6 CFU/ml was used. The volume of 900 µl of WEOO or BEOO and 100 µl of bacterial suspension were mixed in a test tube. Each tube was spotted onto M-H agar after different time exposures (0 min, 5 min, 10 min, 30 min and 60 min) at laboratory temperature. Cultivation was carried out for 24 h at 30 °C under aerobic conditions. Some bacterial strains survived the selected time exposures, therefore, the time exposures were supplemented (3 and 6 h) and the culture period was extended 48–72 h at 30 °C under aerobic conditions. Testing was always performed in duplicate and overall the experiment was independently repeated twice.

3.5.2 *Monitoring of biofilm formation in the presence of oil extracts*

The effect of oil extracts on biofilm formation was studied in 96-well flat-bottomed polystyrene microtiter plates. The microtiter plate contained a positive control for biofilm formation (PK) and a concentration range of oil extracts (0.088–90 %) to which a cell suspension of 1.5×10^8 CFU/ml was added. Determination of biofilm formation was performed identically, see Section 2.3.

4 Results

4.1 *Biofilm formation of Arcobacter-like species on glass and plastic material*

The majority of strains (98.3 %) were confirmed to be able to adhere to the borosilicate surface under at least some experimental conditions. Biofilm formation was affected by the culture atmosphere in a total of 43 strains (72.9 %). Of this number, 38 strains (88.4 %) showed increased biofilm activity under aerobic culture conditions (Table 3 and 4). Based on the results, 27.1, resp. 32.2 % of the isolates were assessed as non-adherent, 30.5, resp. 45.8 % as weakly adherent, 40.7, resp. 20.3 % as moderately adherent, and 1.7, resp. 1.7 % of the strains were categorized as strongly adherent under aerobic and microaerophilic conditions, respectively, after 24 h of exposure. After 72 h of exposure, 8.5, resp. 23.7 % of the strains were assessed as non-adherent, 20.3, resp. 49.2 % as weakly adherent, 67.8, resp. 25.4 % as moderately adherent and 3.4, resp. 1.7 % were categorised as moderately adherent under aerobic or microaerophilic culture conditions, respectively. Another factor observed to influence biofilm formation was exposure time. For example, increased biofilm production under aerobic conditions was observed for *Al. butzleri* UPa 39-3 after both 24 and 72 h of cultivation. Overall, 18.6 and 11.9 % of isolates were categorized as non-adherent, 78.0, resp. 79.7 % as weakly adherent, and 3.4 and 8.5 % were categorized as moderately adherent under aerobic and microaerophilic conditions, respectively, after 24 h of exposure. After 72 h of exposure, 25.4, resp. 5.1 % of the isolates were categorized as non-adherent, 71.2, resp. 88.1 % as weakly adherent, and 3.4, resp. 5.1 % were categorized as moderately adherent under aerobic or microaerophilic conditions, respectively.

4.2 *Biofilm formation of Campylobacter spp. on glass and plastic material*

Four strains (57.1 %) of the total number of biofilm-positive strains showed increased adherence under microaerophilic culture conditions (Table 5). The highest biofilm production on the borosilicate surface was observed for *C. jejuni* CCM 6214 under all experimental conditions. After 24 h of aerobic cultivation, only weak biofilm formation was observed in campylobacter. In the strains *C. jejuni* NS 3668, *C. jejuni* NS 3800 and *C. jejuni* NS 4088, no biofilm formation was observed under the given conditions. After prolonged cultivation (72 h) under aerobic conditions, up to 80.0 % of the strains (except for *C. jejuni* CCM 6214 and *C. jejuni* NS 3668) did not show the ability to form biofilms.

According to the results, 0, resp. 40.0 % of the isolates were assessed as non-adherent and 100, resp. 60.0 % were classified as weakly adherent under aerobic or microaerophilic culture conditions after 24 h of exposure. After 72 h of exposure, 100 and 70.0 % of the isolates were assessed as non-adherent and 0, resp. 30.0 % of the strains were assessed as weakly adherent under aerobic and microaerophilic conditions, respectively. For 10 strains (100 %) of *Campylobacter*, the ability to adhere to the plastic surface was confirmed under at least some experimental conditions. Biofilm formation was also affected by the culture atmosphere in 7 strains (70.0 %), of which 4 strains (57.1 %) showed increased biofilm formation under aerobic culture conditions. All

Campylobacter showed adherence to the plastic surface under aerobic conditions after 24 h of exposure. The results obtained in this case suggest that microaerophilic growing *Campylobacter* show higher biofilm production under adverse atmospheric conditions. However, higher amount of biofilm produced was observed in *C. coli* strain NP 2395 under microaerophilic conditions. Biofilm formation was not observed in most of the strains tested (70.0 %) after 72 h of exposure.

Table 3. Biofilm formation of *Al. butzleri* strains on plastic/glass material at 30 ºC.

Strains	Exposure for 24 h		Exposure for 72 h	
	Aerobic	Microaerophilic	Aerobic	Microaerophilic
A. butzleri UPa 2012/3[A]	W (0.124)/M	W (0.123)/W	W (0.156)/M	W (0.125)/W
A. butzleri UPa 2013/3[B]	W (0.173)/M	M (0.262)/M	W (0.129)/M	W (0.136)/N
A. butzleri UPa 2013/10[A]	N (0.112)/W	N (0.116)/W	N (0.113)/M	W (0.125)/M
A. butzleri UPa 2013/15[A]	N (0.116)/M	W (0.121)/N	W (0.140)/M	W (0.142)/W
A. butzleri UPa 2013/30[B]	W (0.149)/M	W (0.178)/M	W (0.124)/W	W (0.137)/M
A. butzleri UPa 2013/31[B]	W (0.121)/M	W (0.126)/M	W (0.126)/W	W (0.132)/W
A. butzleri UPa 2013/32[B]	W (0.141)/W	W (0.139)/N	W (0.123)/W	W (0.123)/W
A. butzleri UPa 2013/33[B]	N (0.112)/W	N (0.117)/W	W (0.122)/N	W (0.127)/N
A. butzleri UPa 2013/36[A]	W (0.121)/W	W (0.130)/W	N (0.120)/M	W (0.129)/W
A. butzleri UPa 2013/37[B]	W (0.181)/M	W (0.207)/M	W (0.124)/M	W (0.135)/M
A. butzleri UPa 2014/51[B]	W (0.123)/W	W (0.122)/N	W (0.125)/M	W (0.122)/W
A. butzleri UPa 2014/54[B]	N (0.117)/M	N (0.116)/W	W (0.129)/M	W (0.124)/W
A. butzleri UPa 2015/1[B]	W (0.129)/M	W (0.132)/W	N (0.118)/M	W (0.122)/W
A. butzleri UPa 2015/5[A]	W (0.152)/M	W (0.202)/W	N (0.118)/M	W (0.121)/W
A. butzleri UPa 2015/6[B]	W (0.125)/N	W (0.131)/N	N (0.117)/W	W (0.125)/N
A. butzleri UPa 2015/7[B]	M (0.241)/M	W (0.170)/W	W (0.120)/M	W (0.126)/W
A. butzleri UPa 2015/9[B]	W (0.127)/W	W (0.127)/W	N (0.120)/W	W (0.129)/W
A. butzleri UPa 2015/10[B]	W (0.141)/W	W (0.140)/W	W (0.137)/M	W (0.138)/W
A. butzleri UPa 2015/11[B]	W (0.134)/N	W (0.137)/N	W (0.176)/M	W (0.216)/W
A. butzleri UPa 2015/12[B]	W (0.176)/N	W (0.135)/N	N (0.117)/N	W (0.122)/N
A. butzleri UPa 2015/13[B]	N (0.120)/N	W (0.138)/N	W (0.139)/M	W (0.146)/N
A. butzleri UPa 2015/14[B]	W (0.130)/W	W (0.136)/M	W (0.124)/M	W (0.136)/N
A. butzleri UPa 2015/15[B]	W (0.177)/N	W (0.213)/N	W (0.142)/M	W (0.127)/N
A. butzleri UPa 2015/16[B]	W (0.198)/M	M (0.375)/W	W (0.147)/M	W (0.217)/M
A. butzleri UPa 2015/18[B]	W (0.131)/M	W (0.196)/W	N (0.120)/N	W (0.127)/W
A. butzleri UPa 2015/19[B]	W (0.133)/N	W (0.134)/N	W (0.132)/M	W (0.149)/N
A. butzleri UPa 2015/20[C]	W (0.139)/N	W (0.139)/N	W (0.128)/M	W (0.143)/N
A. butzleri UPa 2015/25[B]	M (0.301)/W	W (0.160)/N	W (0.151)/M	W (0.146)/W
A. butzleri UPa KK[B]	N (0.109)/M	N (0.117)/W	N (0.119)/M	W (0.121)/M
A. butzleri UPa 24A[B]	W (0.138)/M	M (0.418)/M	W (0.169)/M	M (0.246)/M
A. butzleri UPa 30B[B]	W (0.131)/W	N (0.118)/N	W (0.137)/M	N (0.118)/W
A. butzleri UPa 39-3[C]	W (0.143)/N	W (0.134)/M	M (0.242)/N	W (0.164)/N
A. butzleri UPa 49B[B]	W (0.130)/W	W (0.128)/N	W (0.124)/M	W (0.122)/W
A. butzleri UPa 65A[B]	W (0.130)/W	W (0.128)/W	W (0.128)/M	W (0.173)/M
A. butzleri UPa 132A[B]	W (0.127)/W	W (0.151)/W	N (0.113)/M	W (0.125)/M
A. butzleri UPa 138A[B]	W (0.141)/M	M (0.265)/W	W (0.122)/M	W (0.153)/W
A. butzleri UPa 141B[B]	W (0.123)/W	W (0.137)/W	N (0.116)/M	W (0.134)/M

Legend: [A] strain isolated from water; [B] strain isolated from food, [C] clinical isolate; UPa–strains isolated at University of Pardubice. N–non-adherent, W–weakly adherent, M–moderately adherent, S–strongly adherent. Value in parentheses represents the actual measured absorbance value.

Table 4. Biofilm formation of *Arcobacter*-like strains on plastic/glass material at 30 ºC.

Strains	Exposure for 24 h		Exposure for 72 h	
	Aerobic	Microaerophilic	Aerobic	Microaerophilic
A. butzleri CCUG 30484	W (0.152)/M	W (0.179)/W	W (0.178)/M	M (0.241)/W
A. butzleri LMG 10828	W (0.126)/M	W (0.130)/W	W (0.132)/M	W (0.178)/W
A. cryaerophilus CCM 3933	N (0.118)/N	N (0.114)/M	N (0.120)/W	W (0.195)/N
A. cryaerophilus CCM 7050	W (0.133)/M	W (0.138)/M	N (0.116)/W	W (0.130)/W
A. defluvii LMG 25694	W (0.228)/S	M (0.379)/S	M (0.414)/S	S (0.504)/S
A. lanthieri LMG 28517	W (0.125)/W	W (0.132)/N	W (0.123)/M	W (0.140)/N
A. skirrovii LMG 6621	W (0.126)/N	W (0.130)/N	W (0.136)/M	W (0.129)/M
A. thereius LMG 24488	W (0.124)/N	W (0.127)/N	N (0.120)/M	W (0.127)/W

Legend: CCUG–strains obtained from Culture Collection University Göteborg, LMG–strains obtained from the Belgian Co-ordinated Collections of Microorganisms, CCM–strains obtained from the Czech Collection of Microorganisms, N–non-adherent, W–weakly adherent, M–moderately adherent, S–strongly adherent. Value in parentheses represents the actual measured absorbance value.

Table 5. Biofilm formation of *Campylobacter*-like strains on plastic/glass material at 30 ºC.

Strains	Exposure for 24 h		Exposure for 72 h	
	Aerobic	Microaerophilic	Aerobic	Microaerophilic
Campylobacter jejuni CCM 6214	W (0.121)/M	N (0.115)/M	N (0.093)/M	N (0.117)/M
Campylobacter jejuni NP 2896[C]	W (0.126)/W	N (0.119)/W	N (0.092)/N	N (0.117)/W
Campylobacter jejuni NS 3668[C]	W (0.125)/N	W (0.127)/N	N (0.114)/W	N (0.117)/W
Campylobacter jejuni NS 3800[C]	W (0.121)/N	W (0.127)/W	N (0.115)/N	W (0.121)/N
Campylobacter jejuni NS 4088[C]	W (0.124)/N	W (0.127)/N	N (0.119)/N	W (0.129)/N
Campylobacter jejuni NS 4091[C]	W (0.124)/W	W (0.125)/N	N (0.113)/N	N (0.118)/N
Campylobacter coli NP 2395[C]	W (0.123)/W	W (0.125)/N	N (0.118)/N	W (0.125)/N
Campylobacter coli NS 3803[C]	W (0.121)/W	N (0.116)/N	N (0.094)/N	N (0.116)/N
Campylobacter coli NS 4062[C]	W (0.121)/W	N (0.115)/N	N (0.094)/N	N (0.118)/W
Campylobacter sp. ONN 366[C]	W (0.132)/W	W (0.130)/N	N (0.115)/N	N (0.118)/W

Legend: [C] clinical isolate; CCM–strains obtained from the Czech Collection of Microorganisms, NP–strains obtained from Pardubice Hospital, NS–strains obtained from Svitavy Hospital, ONN–strains obtained from Náchod Regional Hospital, N–non-adherent, W–weakly adherent, M–moderately adherent, S–strongly adherent. Value in parentheses represents the actual measured absorbance value.

4.3 *Effect of antibiotics on Arcobacter-like species*

4.3.1 *Effect of antibiotics on planctonic cells of Arcobacter-like species*

The susceptibility of 60 strains of *Arcobacter*-like species to selected antimicrobial agents was monitored in the experiments (Table 6). *Arcobacter*-like species are mostly sensitive to macrolide antibiotics, aminoglycosides and quinolones. The results showed that 47 of 60 (78.3 %) strains were sensitive to ampicillin, 57 of 60 (95.0 %) strains to ciprofloxacin, 7 of 60 (11.7 %) strains to clindamycin, 60 of 60 (100 %) strains to erythromycin, 59 of 60 (98.3 %) strains to gentamicin and 24 of 60 (40.0 %) strains to tetracycline. Erythromycin (MIC_{50}/MIC_{90} of 2 mg/l and 4 mg/l, respectively) was evaluated as the most effective antibiotic to suppress *Arcobacter*-like species. mg/l), gentamicin (*Al. butzleri* strains – MIC_{50}/MIC_{90} 0.25 mg/l and 1 mg/l, respectively; *Al. cryaerophilus* strains – MIC_{50}/MIC_{90} 0.5 mg/l and 1 mg/l, respectively) and ciprofloxacin (*Al. butzleri* strains – MIC_{50}/MIC_{90} 0.06 mg/l and 0.13 mg/l, respectively) were evaluated as the most effective antibiotics against *Arcobacter*-like

species. At the same time, high level of multidrug resistance was observed. Nine strains (15.0 %) showed resistance to a combination of three antibiotics – in seven cases it was a combination of ampicillin, clindamycin and tetracycline, in two cases it was a combination of ciprofloxacin, clindamycin and tetracycline (Table 7).

Diseases caused by *Arcobacter*-like species can in some cases be treated by the administration of appropriate antibiotics. Many studies have recommended tetracyclines and aminoglycosides as the first choice antibiotics in the treatment of arcobacteriosis (Aski et al., 2016; Rathlavath et al., 2017). According to our results, ciprofloxacin (quinolones) could be used to treat arcobacteriosis. Numerous susceptibility of *Arcobacter*-like species to ciprofloxacin has been reported in other studies (Houf et al., 2004; Rathlavath et al., 2017).

Table 6. MIC and MCB data on antibiotic agents determined for *Arcobacter*-like strains.

	Number of isolates susceptible at given concentrations and MICs (mg/L)																
	0.03	0.06	0.13	0.25	0.5	1	2	4	8	16	32	64	128	256	MIC_{50}	MIC_{90}	R
Ampicillin																	
A. butzleri strains (50)			1	3	2	1	4	5	15	7	6	5	1		8	64	24%
A. cryaerophilus strains (3)									1	2					8	128	0%
Ab CCUG 30484										1							
Ab LMG 10828													1				
Ac CCM 3933										1							
Ac CCM 3934								1									
Ac CCM 7050								1									
Ad LMG 25694								1									
As LMG 6621										1							
Ciprofloxacin																	
A. butzleri strains (50)	20	13	12	3							1	1			0.06	0.13	4%
A. cryaerophilus strains (3)	1		1									1			0.13	64	33.3%
Ab CCUG 30484		1															
Ab LMG 10828			1														
Ac CCM 3933		1															
Ac CCM 3934		1															
Ac CCM 7050			1														
Ad LMG 25694		1															
As LMG 6621		1															
Clindamycin																	
A. butzleri strains (50)		2		1	3	1	2	2	5	7	15	6	6		32	128	86%
A. cryaerophilus strains (3)								1		1	1				16	32	100%
Ab CCUG 30484												1					
Ab LMG 10828														1			
Ac CCM 3933									1								
Ac CCM 3934										1							
Ac CCM 7050												1					
Ad LMG 25694								1									
As LMG 6621								1									
Erythromycin																	
A. butzleri strains (50)		1		3	4	8	13	17	4						2	4	0%
A. cryaerophilus strains (3)						1	1	1							2	4	0%
Ab CCUG 30484					1												
Ab LMG 10828									1								
Ac CCM 3933							1										
Ac CCM 3934							1										
Ac CCM 7050							1										
Ad LMG 25694						1											
As LMG 6621				1													
Gentamicin																	
A. butzleri strains (50)		12		22	9	6	1								0.25	1	0%
A. cryaerophilus strains (3)				1	1	1									0.5	1	0%
Ab CCUG 30484					1												
Ab LMG 10828									1								
Ac CCM 3933						1											
Ac CCM 3934				1													
Ac CCM 7050				1													
Ad LMG 25694		1															
As LMG 6621					1												
Tetracycline																	
A. butzleri strains (50)				3	2	8	10	7	5	7	4	1	3		4	64	54%
A. cryaerophilus strains (3)							1			1	1				16	32	66.7%
Ab CCUG 30484													1				
Ab LMG 10828													1				
Ac CCM 3933												1					
Ac CCM 3934									1								
Ac CCM 7050									1								
Ad LMG 25694										1							
As LMG 6621										1							

Legend: *Ab–Al. butzleri*; *Ac– Al. cryaerophilus*; *Ad–Pseudabacter defluvii*; *Al–Al. lanthieri*; *At–Al. thereius.* Resistance breakpoints were applied to the CLSI for *C. coli* and *Enterobacteriaceae.* Grey shading indicates resistant strains. R – percentage of resistant strains.

Table 7. Multi-drug resistence of *Arcobacter*-like strains.

Arcobacter-like strain	Resistance patterns						
	AMP, CLI	AMP, TET	CLI, TET	AMP, CLI, TET	CIP, CLI, TET	AMP, CIP, CLI, TET	AMP, CLI, GEN, TET
A. butzleri strains (50)	3	1	17	6	1	1	
A. cryaerophilus strains (3)				1	1		
Ab CCUG 30484			1				
Ab LMG 10828							1
Ac CCM 3933			1				
Ac CCM 3934			1				
Ac CCM 7050			1				
Ad LMG 25694			1				
As LMG 6621			1				

Legend: AMP–ampicilin; CIP–ciprofloxacin; CLI–clindamycin; GEN–gentamicin; TET–tetracycline.

4.3.2 *Effect of antibiotics on biofilm formation of Arcobacter-like species*

All strains showed the ability to form biofilms, but the intensity of biofilm formation varied between strains (Figure 1). Higher biofilm formation capacity was observed especially in strains in environments with lower concentrations (usually 0.13–2 mg/l) of antibiotics. This can be explained by the stressful environmental conditions to which the bacteria responded by switching to a more resistant biofilm form. The results suggest that antibiotic concentrations below MIC values could significantly affect bacterial biofilm formation. For example, a significant decrease in biofilm formation occurred in *Al. butzleri* strain CCUG 30484 in ampicillin medium at a concentration equal to or greater than 2 mg/l and in *Al. cryaerophilus* UPa 2013/13 at a clindamycin concentration equal to or greater than 2 mg/L. Biofilm formation of *Al. butzleri* UPa 2015/14 was reduced at an ampicillin concentration of 16 mg/l, which corresponds exactly to the MIC established for planctonic cells of the same strain. Ciprofloxacin was evaluated as the most effective antibiotic suitable to inhibit biofilm formation of *Arcobacter*-like species.

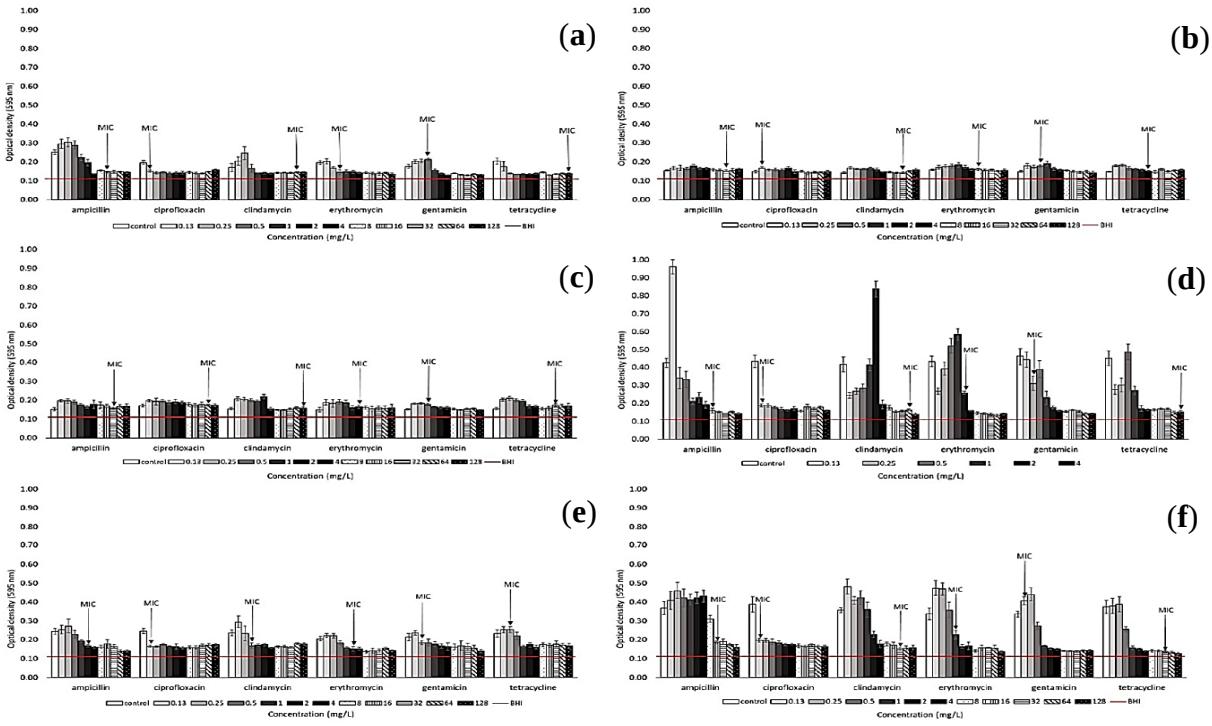

Figure 1. Biofilm formation in the presence of antibiotics. (**a**) *Al. butzleri* CCUG 30484; (**b**) *Al. butzleri* UPa 2013/6; (**c**) *Al. butzleri* UPa 2013/9; (**d**) *Al. butzleri* UPa 2013/30; (**e**) *Al. butzleri* UPa 2015/13; (**f**) *Al. butzleri* UPa 2015/14. The horizontal line represents the influence of BHI broth (values under horizontal line-biofilm-negative; values above line-biofilm-positive).

4.4 *Effect of hydrosols on Arcobacter-like species*

Using chemical analyses, 48 compounds were identified in the lavender hydrosols. The largest difference in the chemical composition of the lavender hydrosols was observed in the linalool content (23.2% and 7.9% in H_{HD_SPE} and H_{SD_SPE}, respectively). The dominant compound in lavender hydrosols was 1,8-cineole, which is often described as an important substance in lavender oils (Politi et al., 2020). A total of 33 compounds were identified in both types of laurel hydrosols. The majority compound was 1,8-cineole, in both types of hydrosols. The dominant compounds of the fennel hydrosols were estragole and fenchone, which accounted for almost 60 % of all identified compounds. The main identified compounds in clove hydrosols were phenolic derivatives, specifically eugenol (92.7 % in H_{HD_SPE}, 89.1 % in H_{SD_SPE}) and eugenyl acetate (9.4 % in H_{HD_SPE}, 5.6 % in H_{SD_SPE}).

4.4.1 *Antimicrobial effect of hydrosols*

No strain of *Arcobacter*-like species was suppressed by non-concentrated hydrosols derived from lavender, fennel or laurel. However, clove hydrosols showed weak antimicrobial activity against *Al. thereius* LMG 24488 strain (H_{HD}, zone of inhibition 6.5±0.3 mm; H_{SD}, zone of inhibition 6.8±0.4 mm).

Hydrosols after their concentration by SPE showed significant antimicrobial aktivity (Table 8). Concentrated clove hydrosols showed the highest antimicrobial activity among all the samples tested. Lavender hydrosol obtained by steam distillation showed the strongest antimicrobial activity against *Al. butzleri* CCUG 30484 and *Al. thereius* LMG 24488 (13.5±0.6 and 13.3±0.9 mm inhibition zone, respectively). In contrast, the growth of *Al. butzleri* UPa 2012/3 strain was more inhibited by lavender hydrosol obtained by hydrodistillation (12.8±0.3 mm inhibition zone). The highest resistance of *Arcobacter*-like species was observed in the case of fennel hydrosol.

Table 8. Antimicrobial activity of hydrosols concentrated by SPE on *Arcobacter*-like strains–mean inhibition zones.

			Ab LMG 10828	*Ab* CCUG 30484	*Ab* UPa 2012/3	*Ac* CCM 7050	*Ac* UPa 2013/13	*Al* LMG 28517	*As* LMG 6621	*At* LMG 24488
Lavender	IZ	H_{HD_SPE}	9.5 ± 0.3	8.8 ± 0.3	12.8 ± 0.3	8.3 ± 0.5	8.0 ± 0	11.3 ± 1.0	9.0 ± 0.8	11.5 ± 0.3
		H_{SD_SPE}	10.8 ± 0.3	13.5 ± 0.6	10.5 ± 0.3	9.5 ± 0.9	11.5 ± 0.1	11.8 ± 0.3	10.3 ± 0.5	13.3 ± 0.9
	MIC/ MBC	H_{HD_SPE}	0.4/0.8	0.8/0.8	0.8/1.6	0.8/0.8	0.8/0.8	0.8/0.8	0.4/0.4	0.8/1.6
		H_{SD_SPE}	0.4/0.4	0.4/0.8	1.6/1.6	0.4/0.8	0.4/0.4	0.4/0.8	0.4/0.4	0.4/0.8
Fennel	IZ	H_{HD_SPE}	7.8 ± 0.2	9.0 ± 0.8	10.5 ± 0.9	9.8 ± 0	8.5 ± 1.3	10.8 ± 0.5	7.8 ± 0.5	12.0 ± 0.1
		H_{SD_SPE}	10.8 ± 0	10.8 ± 0.5	10.8 ± 0.9	10.0 ± 0.2	10.0 ± 0.8	11.8 ± 0	10.3 ± 0.3	11.3 ± 0.5
	MIC/ MBC	H_{HD_SPE}	1.6/1.6	3.1/3.1	1.6/3.1	0.8/0.8	1.6/1.6	1.6/1.6	0.8/0.8	1.6/1.6
		H_{SD_SPE}	0.8/1.6	1.6/3.1	1.6/1.6	0.4/0.8	0.8/0.8	0.8/1.6	0.4/0.4	0.8/0.8
Bay leaves	IZ	H_{HD_SPE}	8.0 ± 0.2	9.0 ± 0.8	9.0 ± 0	9.5 ± 0.3	9.5 ± 0.3	11.3 ± 0.3	9.0 ± 0.3	10.8 ± 0
		H_{SD_SPE}	10.5 ± 0.3	9.5 ± 0.6	14.0 ± 0.8	10.3 ± 0.3	10.0 ± 0.3	10.5 ± 0.6	12.5 ± 0.7	13.3 ± 0.6
	MIC/ MBC	H_{HD_SPE}	1.6/1.6	1.6/1.6	1.6/3.1	0.8/1.6	0.8/1.6	1.6/3.1	1.6/3.1	1.6/1.6
		H_{SD_SPE}	0.8/1.6	0.8/1.6	0.8/1.6	0.8/1.6	0.8/1.6	1.6/3.1	1.6/1.6	0.8/0.8
Clove	IZ	H_{HD_SPE}	10.5 ± 0.3	12.0 ± 0.9	13.0 ± 0.6	10.5 ± 0.3	14.3 ± 0.5	12.0 ± 0.6	12.0 ± 0.9	14.8 ± 0.5
		H_{SD_SPE}	12.5 ± 0.7	13.5 ± 0.4	14.5 ± 0.3	12.5 ± 0.3	16.5 ± 0.3	12.8 ± 0.7	11.0 ± 0.3	15.5 ± 0.7
	MIC/ MBC	H_{HD_SPE}	0.1/0.1	0.2/0.2	0.8/0.8	0.2/0.2	0.4/0.4	0.4/0.4	0.2/0.2	0.4/0.4
		H_{SD_SPE}	0.1/0.1	0.1/0.1	0.8/0.8	0.1/0.1	0.1/0.1	0.4/0.4	0.1/0.1	0.2/0.4
Control	IZ		8.0 ± 0.8	7.7 ± 0.5	7.7 ± 0.5	8.0 ± 0.8	7.0 ± 0.8	6.7 ± 0.5	8.3 ± 0.5	8.0 ± 0.8
	MIC/MBC		3.1/6.3	3.1/6.3	3.1/6.3	1.6/3.1	3.1/3.1	3.1/3.1	1.6/3.1	3.1/3.1

Legend: H_{HD_SPE}–hydrolate obtained by hydrodistillation and 50× concentrated using solid phase extraction (SPE); H_{SD_SPE}–hydrolate obtained by steam distillation and 50× concentrated using SPE; Control–solvent without active compounds; IZ–inhibition zone; MIC–minimal inhibitory concentration; MBC–minimal bactericidal concentration; *Ab–A. butzleri*; *Ac–A. crvaerophilus*; *Al–A. lanthieri*; *As–A. skirrowii*; *At–A. thereius*.

4.4.2 *Biofilm formation in the presence of hydrosols*

Some strains showed a significant decrease in the amount of biofilm formed in the presence of unconcentrated hydrosols (Figure 2). For example, the biofilm formation of *Al. thereius* and *Al. lanthieri* strains decreased by up to 35.9 % in the presence of hydrosol from laurel obtained by steam distillation. In fact, in the case of concentrated hydrosols (Figure 2), some of the strains tested showed increased biofilm formation compared to the amount of biofilm formed in the environment without the hydrosol sample. A rapid increase in biofilm formation capacity was observed in the H_{SD_SPE} clove medium, for example, in the strains *Al. lanthieri* LMG 28517 and *Al. thereius* LMG 24488 (H_{HD_SPE} clove hydrosol). However, biofilm formation of *Al. butzleri* CCUG 30484, *Al. cryaerophilus* CCM 7050 and *S. aureus* CCM 4223 was reduced (compared to other strains) in the environment of concentrated hydrosols from lavender, fennel and laurel.

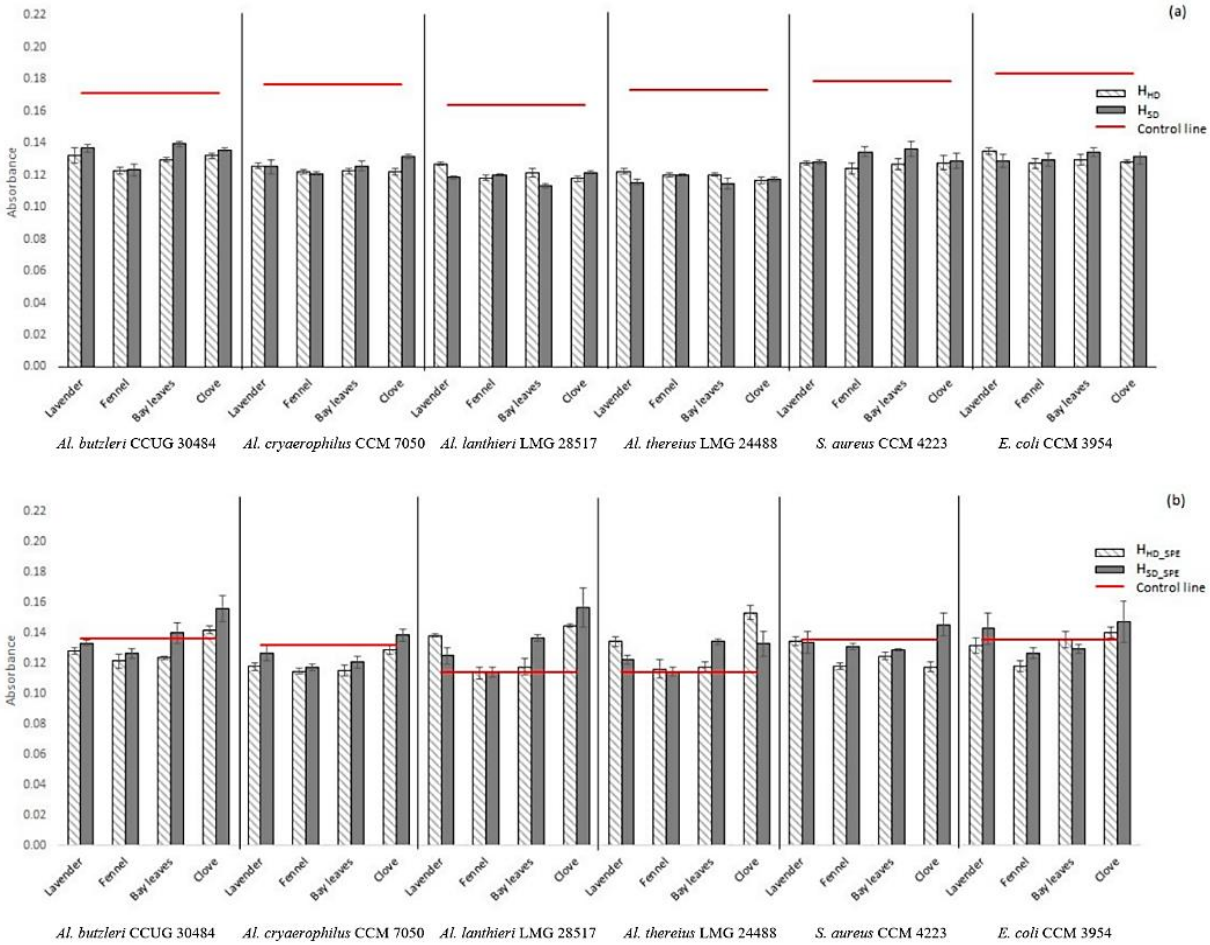

Figure 2. Biofilm formation in the presence of non-concentrated hydrolates (**a**) and concentrated hydrolates (**b**). Bars represent standard deviation, n = 3. H_{HD}–hydrolate obtained by hydrodistillation; H_{SD}–hydrolate obtained by steam distillation; H_{HD_SPE}–hydrolate obtained by hydrodistillation and 50× concentrated using solid phase extraction (SPE); H_{SD_SPE}–hydrolate obtained by steam distillation and 50× concentrated using SPE. Red lines–biofilm formation of strains in water (**a**), biofilm formation of strains in extraction solvent (**b**); values above/below the red lines–increased/reduced biofilm formation due to hydrolate presence.

4.5 *Effect of oil extracts on Arcobacter-like species*

The analysis revealed that hydroxytyrosol, tyrosol, isomers and oleuropein derivatives are the main constituents of buffered and unbuffered aqueous extracts. The individual compounds were identified by comparison with the retention times and voltammetric profiles of the measured standard compounds. The hydroxytyrosol and tyrosol contents were determined by the calibration curve method. Isomers and derivatives of oleuropein were expressed as sum of peaks and their content was related to oleuropein as standard. Limits of detection (LOD) and limits of quantification (LOQ) were determined for each compound. Baseline noise was always evaluated from five injections of a blank experiment (mobile phase A).

Table 9. Contents of followed compounds (mg/l) in WEOO (non-buffered) and BEOO (buffered).

	Chemical Compound	Extra-Virgin Olive Oil (Ballester)	Extra-Virgin Olive Oil (Kyosos)	Blended Olive and Sunflower Oil (Ondoliva)	Refined Olive Oil (Borges)	Olive Pomace Oil (Ondoliva)
WEOO	Hydroxytyrosol	9.82 ± 0.12^{a}	9.01 ± 0.24^{b}	0.40 ± 0.01^{c}	<LOD	<LOD
	Tyrosol	8.93 ± 0.12^{a}	10.17 ± 0.06^{b}	<LOQ	<LOD	<LOD
	Oleuropein derivates	52.64 ± 1.84^{a}	29.20 ± 1.95^{b}	0.99 ± 0.15^{c}	<LOD	<LOD
BEOO	Hydroxytyrosol	9.97 ± 1.81^{a}	7.97 ± 0.08^{b}	0.93 ± 0.08^{c}	<LOD	<LOD
	Tyrosol	12.30 ± 2.04^{a}	12.49 ± 0.72^{a}	2.70 ± 0.07^{b}	<LOD	<LOD
	Oleuropein derivates	10.26 ± 0.44^{a}	7.69 ± 0.01^{b}	0.79 ± 0.15^{c}	<LOD	<LOD

Legend: The results are presented as a mean ± standard deviation. Statistical significance between the groups was assessed by ANOVA at 5% level. Means in the same row with different superscript small letters differ significantly ($p < 0.05$). LOD–limit of detection; LOQ–limit of quantification.

The total polyphenol content was measured spectrophotometrically with Folin-Ciocalteu reagent and the results were expressed as gallic acid equivalent (GAE in mg/L). The extra virgin olive oil extracts showed the highest total polyphenol content (43.50–93.23 mg/L), followed by the mixed sunflower and olive oil, olive pomace oil and refined olive oil samples. These results correspond with the phenolic compounds detected by HPLC-CoulArray (Figure 3). Specifically, 71.4 mg/l of active compounds were detected in Spanish extra virgin olive oil and 48.3 mg/l in Greek extra virgin olive oil (Table 9). In mixed olive and sunflower oil as well as olive pomace oil, the bioactive substances of interest were not detected at all or were below the LOD.

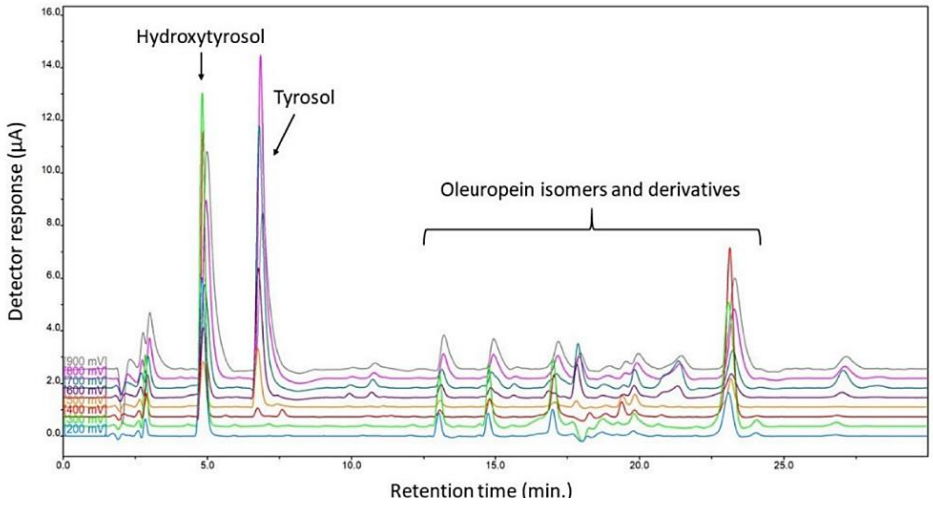

Figure 3. Chromatogram of non-buffered extract of Spanish extra virgin olive oil. Chromatographic conditions – Gemini C18 column (150 mm×3 mm, 3 µm). Mobile phase A–5 mM ammonium acetate with formic acid (pH~3); mobile phase B–acetonitrile; flow rate 0.4 ml/min; gradient elution–0–30 min, 5–60% of mobile phase B; sample volume 10 µl; temperature 40 ºC; detection at potentials 200–900 mV.

4.5.1 *Effect of oil extracts on planctonic cells*

The strongest antimicrobial effect was observed for buffered extracts of extra virgin olive oil (Figure 4). The extra virgin olive oil extract fully inhibited *Al. butzleri* UPa 2013/30, *Al. cryaerophilus* CCM 7050 and *Al. cryaerophilus* UPa 2013/13 strains after only 30 min of exposure. A slightly lower antimicrobial effect was observed for the Greek extra virgin olive oil extract. Other oil extracts showed lower antimicrobial efficacy compared to extra virgin olive oil extracts. In the case of refined olive oil extracts, complete inhibition of most of the tested strains was observed only after 24 h of exposure. An exception was *Al. thereius* LMG 24488, where a decrease in viable cells was observed after 60 min exposure and complete inhibition occurred after 6 h exposure. The cake oil extract showed the lowest antimicrobial efficacy compared to all other buffered oil extracts.

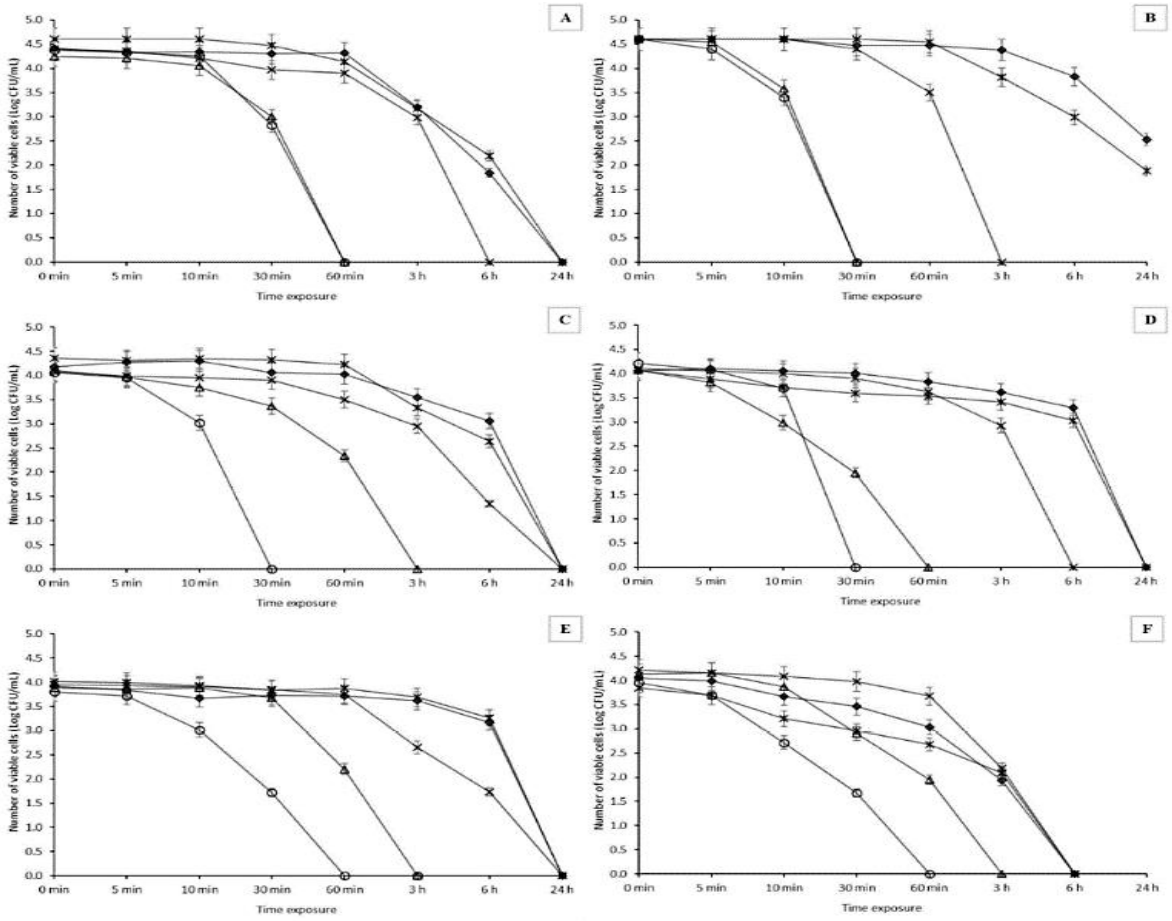

Figure 4. Survival of *Arcobacter*-like strains in the presence of buffered extracts at a sample concentration of 90%. Spanish extra-virgin olive oil (circle); Greek extra-virgin olive oil (triangle); blended sunflower and extra-virgin olive oil (times); pure olive oil (diamond); olive pomace oil (asterisk). (**A**) – *Al. butzleri* CCUG 30484; (**B**) – *Al. butzleri* UPa 2013/30; (**C**) – *Al. cryaerophilus* CCM 7050; (**D**) – *Al. cryaerophilus* UPa 2013/13; (**E**) – *Al. lanthieri* LMG 28517; (**F**) – *Al. thereius* LMG 24488. The results are presented as a mean ± standard deviation.

The aqueous extract of extra virgin olive oil showed the highest antimicrobial efficacy (Figure 5). This oil extract was able to inhibit most of the strains studied after only 5 min of exposure. The refined oil extract very strongly inhibited *Al. thereius* LMG 24488 and *Al. cryaerophilus* CCM 7050 strains. The lowest antimicrobial effect

was observed for the olive pomace oil extract, with complete inhibition occurring only after 3–24 h of exposure. For example, for *Al. lanthieri* strain LMG 28517, there was almost no decrease in viable cells during 6 h of exposure.

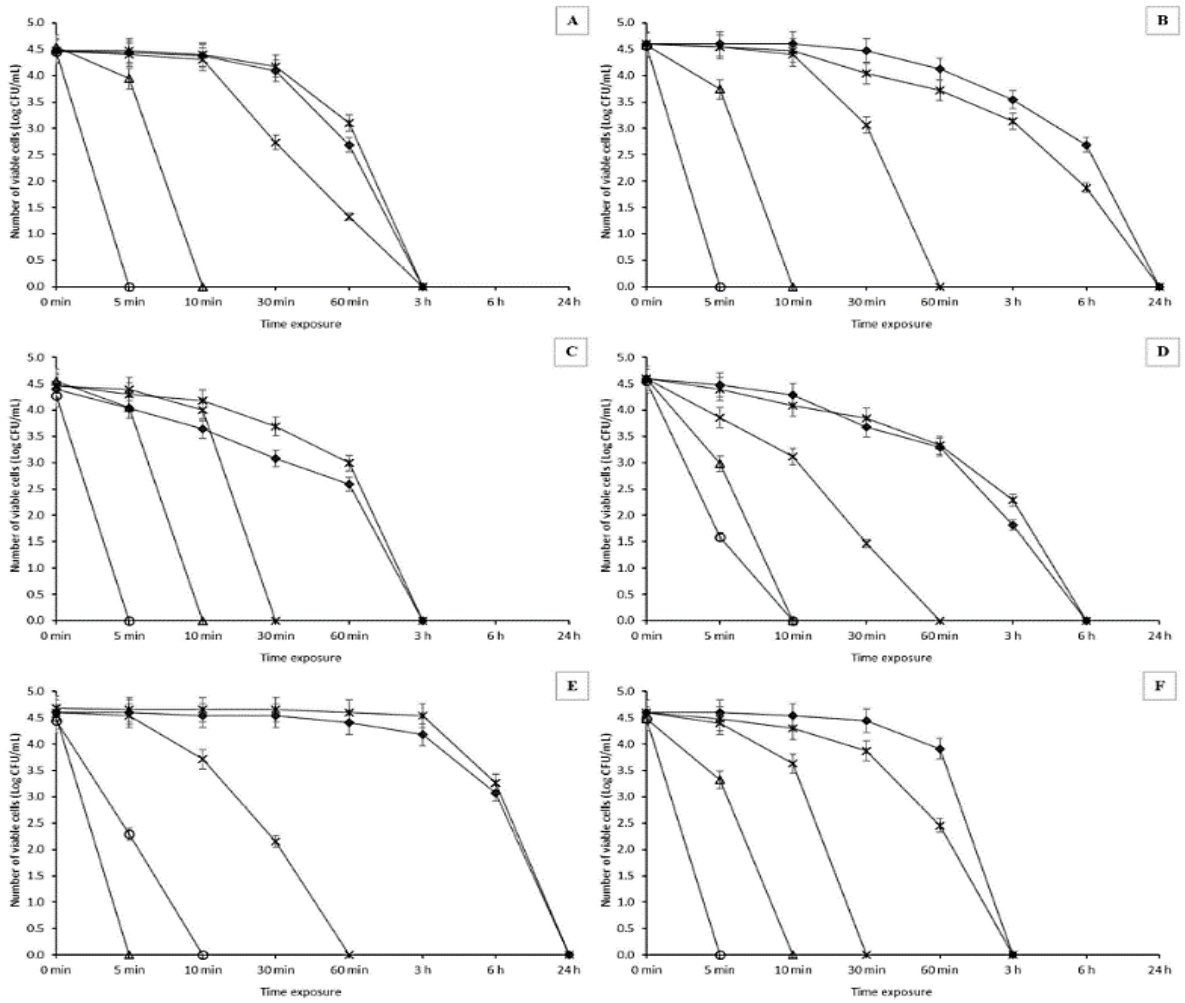

Figure 5. Survival of *Arcobacter*-like strains in the presence of non-buffered extracts at a sample concentration of 90%. Spanish extra-virgin olive oil (circle); Greek extra-virgin olive oil (triangle); blended sunflower and extra-virgin olive oil (times); pure olive oil (diamond); olive pomace oil (asterisk). (**A**) – *Al. butzleri* CCUG 30484; (**B**) – *Al. butzleri* UPa 2013/30; (**C**) – *Al. cryaerophilus* CCM 7050; (**D**) – *Al. cryaerophilus* UPa 2013/13; (**E**) – *Al. lanthieri* LMG 28517; (**F**) – *Al. thereius* LMG 24488. The results are presented as a mean ± standard deviation.

The results obtained from the chemical analyses confirmed that the extra virgin olive oil extracts contained the most bioactive compounds, which was confirmed by testing the antimicrobial activity of the oil extracts. The highest antimicrobial effect was observed for extra virgin olive oil extracts and the lowest antimicrobial effect was observed for cake olive oil extract. The antimicrobial effects of olive oils are higher than those of other vegetable oils, probably due to the high content of fatty acids that contribute to the antimicrobial activity (Medina et al., 2006). Phenolic compounds are the main source of compounds with antimicrobial effects in olive oils, as confirmed by many other studies (Friedman et al., 2003; Furneri et al., 2004).

4.5.2 *Effect of oil extracts on biofilm formation*

It can be concluded that the highest concentration of extracts tested usually led to increased biofilm formation. This is probably due to the increased stress acting on the planctonic cells, which could no longer survive in this form and immediately focused on bacterial biofilm formation. Biofilm formation of *Al. butzleri* and *Al. cryaerophilus* strains was comparable in the presence of buffered extracts (Figure 6). However, at higher concentrations of the extract, there was a significant decrease in biofilm formation. In the case of the refined olive oil extract, biofilm formation was increased in almost the entire concentration range.

The pH of the prepared extracts, which was not modified in any way, also played an important role. The pH value of non-buffered extracts was in the basic range (pH ~ 8.73) and thus may have an effect on biofilm formation. For example, biofilm formation of *Al. butzleri* UPa 2013/30 was increased many fold in the environment of aqueous extra virgin olive oil extract and Greek extra virgin olive oil (Figure 7). Increased biofilm formation of up to 50 % was observed in this strain compared to biofilm formation without the influence of extracts. It can be observed that in 90 % of the extracts there was a significant reduction in biofilm formation, sometimes these values reached to the limit of positivity of biofilm formation.

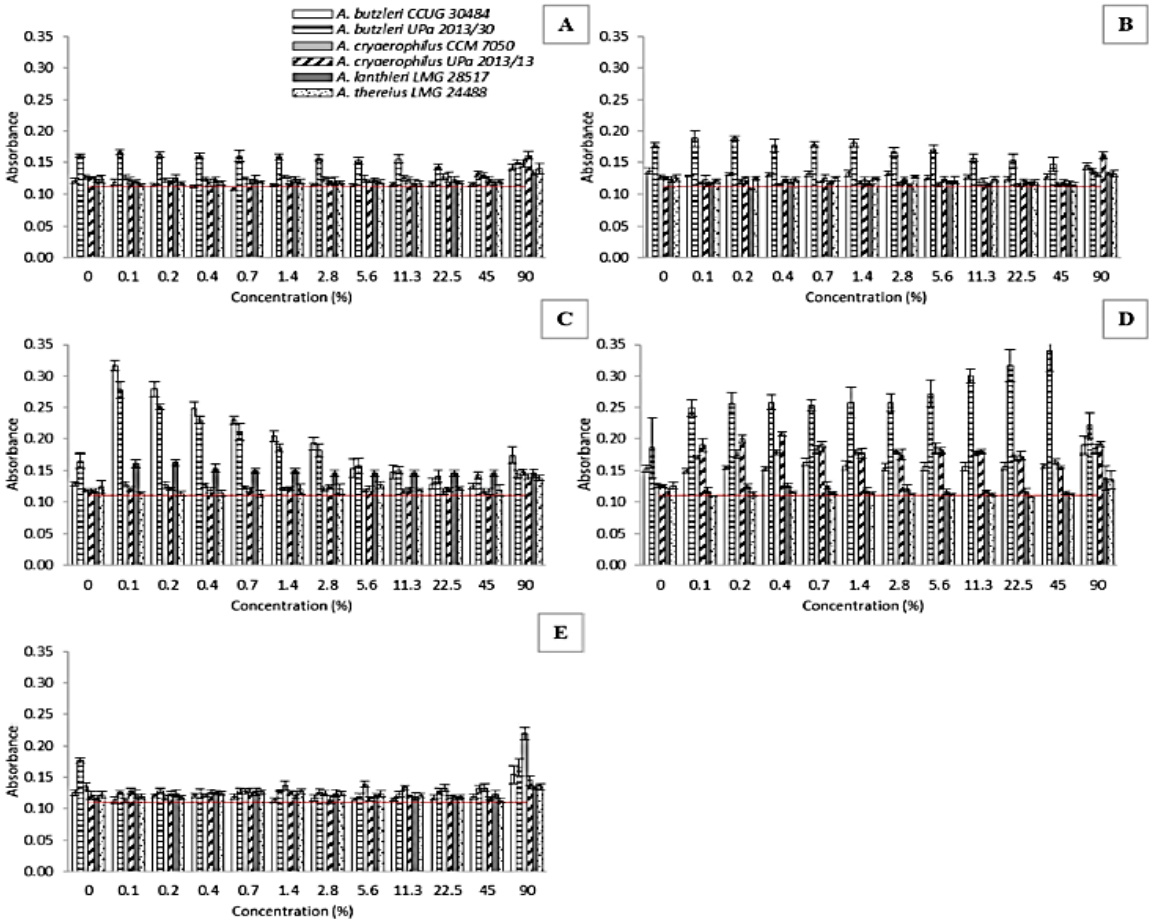

Figure 6. Biofilm formation in the presence of buffered extracts at a concentration of 90%. (**A**)–extra virgin olive oil (Ballester); (**B**)–extra virgin olive oil (Kyosos); (**C**)–blended sunflower and extra-virgin olive oil (Ondoliva); (**D**)–pure olive oil (Borges); (**E**)–olive pomace oil (Ondoliva). The horizontal red line represents the influence of BHI broth (values under horizontal line-biofilm negative; values above line-biofilm positive). The results are presented as a mean ± standard deviation.

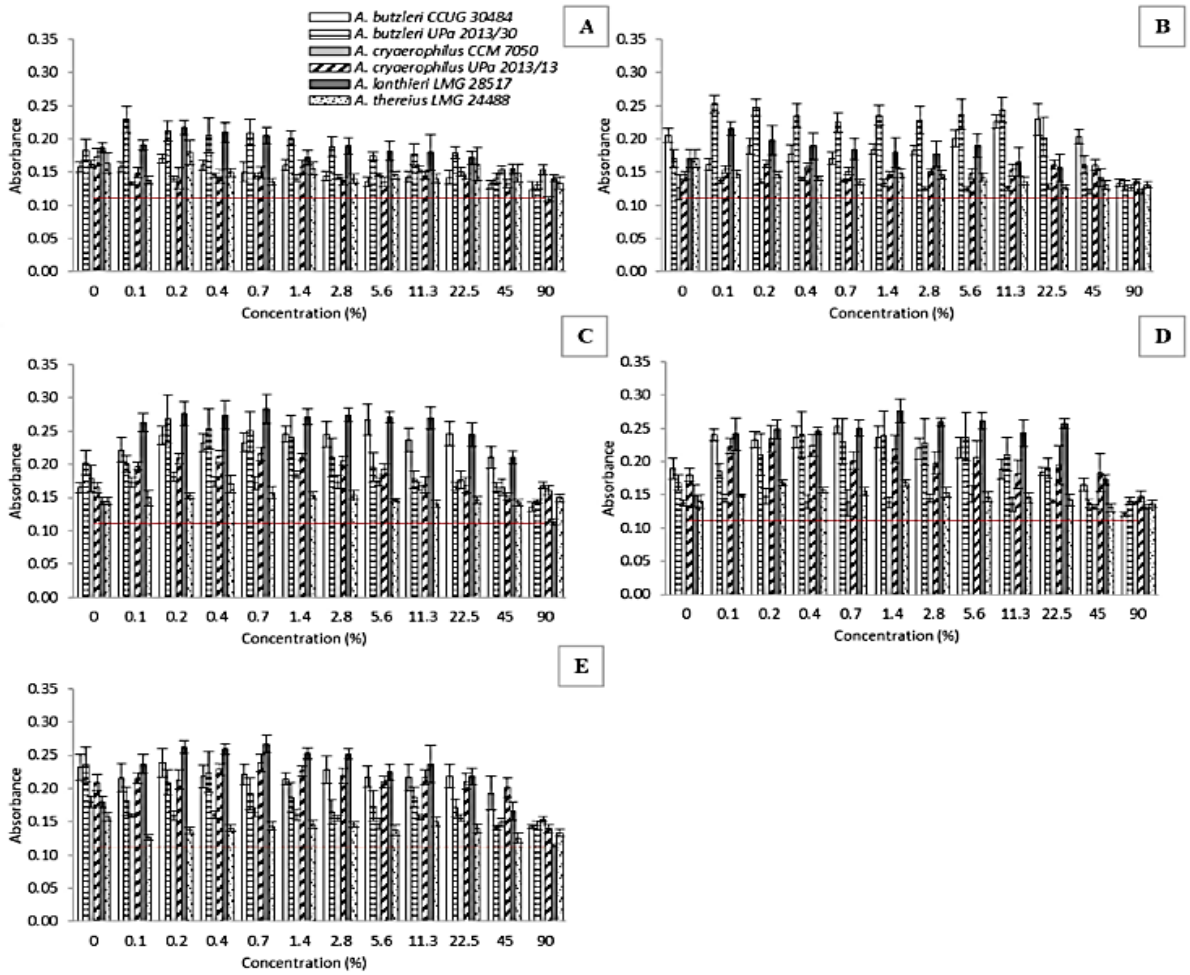

Figure 7. Biofilm formation in the presence of non-buffered extracts at a concentration of 90%. (**A**)–extra virgin olive oil (Ballester); (**B**)–extra virgin olive oil (Kyosos); (**C**)–blended sunflower and extra-virgin olive oil (Ondoliva); (**D**)–pure olive oil (Borges); (**E**)–olive pomace oil (Ondoliva). The horizontal red line represents the influence of BHI broth (values under horizontal line-biofilm negative; values above line-biofilm positive). The results are presented as a mean ± standard deviation.

5 Conclusion

Arcobacter-like species are among the potentially dangerous bacteria and therefore it is advisable to monitor their occurrence. Efforts should also be made to understand the mechanisms of pathogenicity and where appropriate, ways to influence the survival of these bacteria in both planktonic and biofilm form. Resistance to antimicrobials is a threat, particularly in cases where biofilms are involved, as bacteria in biofilms are many times more resistant than planktonic cells.

The main focus of this study was to evaluate the biofilm formation of the tested microorganisms. Bacterial biofilm formation was monitored in 69 strains of the *Campylobacteraceae* and *Arcobacteraceae* families. The amount of biofilm formed was evaluated in aerobic and microaerophilic atmosphere after 24 or 72 h of cultivation on different culture surfaces. The results show that the strains differ significantly in the intensity and the actual biofilm formation. The different bacterial species also differ in their requirements to the cultivation conditions and thus in the quantity and quality of biofilm formed. It is therefore not possible to say unequivocally which conditions are best for biofilm formation. For example, in the case of *Pd. defluvii* LMG 25694, an increase in biofilm of more than 80.0% was observed after 72 h of cultivation in an aerobic environment. However, most strains did not show differences in biofilm production after 24 or 72 h of exposure.

Another objective of this study was to evaluate the effects of several types of antimicrobial agents on planktonic cells and biofilm formation by *Arcobacter*-like species. Among the tested agents with antimicrobial potential, commercially available antibiotics, hydrosols obtained by steam distillation or hydrodistillation from four plant matrices, and oil extracts obtained by extraction from olive oils of different quality were selected.

A total of 60 *Arcobacter*-like strains were monitored for susceptibility to the selected antibiotics. The results confirmed that *Arcobacter*-like species were sensitive to macrolides, aminoglycosides and quinolones. Erythromycin, gentamicin and ciprofloxacin were found to be the most effective antibiotics for suppressing *Arcobacter*-like species. In contrast, many strains showed significant resistance to lincosamides, e.g. 88.3 % of strains were resistant to clindamycin. No strain showed resistance to erythromycin. However, a high level of multidrug resistance was confirmed. Of the strains tested, 15.0 % showed resistance to a combination of three antibiotics, the most common being a combination of ampicillin, clindamycin and tetracycline. Higher biofilm formation was also observed in strains in the presence of lower antibiotic concentrations. There was a decrease in biofilm formation with higher concentration of antibiotics. The greatest suppression of biofilm formation of *Arcobacter*-like species was observed with ciprofloxacin.

In lavender hydrosols, 1,8-cineole and linalool were the most abundant compounds. Similarly, in hydrosols of laurel, the dominant compound in both types of hydrosols was 1,8-cineole. The main compounds in fennel hydrosols, estragole and fenchone, accounted for almost half of the content of fennel hydrosols. The most abundant compounds identified in clove hydrosols were eugenol and eugenyl acetate. The antimicrobial efficacy of the unconcentrated and concentrated hydrosols was evaluated against 8 *Arcobacter*-like species as well as Staphylococcus aureus CCM 4223, *Enterococcus faecalis* CCM 4224, *Escherichia coli* CCM 3954, *Pseudomonas*

aeruginosa CCM 3955 and *Candida albicans* CCM 8186 using the disc diffusion method. No antimicrobial activity was observed for most of the tested unconcentrated H_{HD} and H_{SD} hydrosols against the studied microorganisms, while the concentrated hydrosols showed significant antimicrobial activity. Resistance of *Arcobacter*-like species was observed especially to fennel hydrosol. Biofilm formation of strains in the hydrosol environment was not significantly affected in the case of non-concentrated hydrosols significantly results, however, a rapid increase in biofilm formation was observed for concentrated hydrosols. The greatest increase in biofilm formed was observed in the clove hydrosol environment.

Furthermore, the viability of *Arcobacter*-like species was monitored in unbuffered (WEOO) and buffered (BEOO) aqueous extracts of olive oils. Olive oil extracts of different quality were analyzed by HPLC with electrochemical CoulArray detection. The main components detected were hydroxytyrosol, tyrosol, isomers and oleuropein derivatives. The aqueous extracts of extra virgin olive oils contained the highest amount of bioactive compounds. Spanish extra virgin olive oil contained 71.4 mg/l of bioactive substances and Greek extra virgin olive oil contained 48.3 mg/l of bioactive substances. The other extracts showed very low levels of the monitored bioactive substances, with two oils having all monitored bioactive substances below the LOD. The strongest antimicrobial effect was observed for the extra virgin olive oil extracts, which corresponds with the results of the chemical composition of the prepared extracts. *Arcobacter*-like species were inactivated by buffered Spanish extra virgin olive oil extracts after only 30 min of exposure. A slightly lower antimicrobial effect was observed for the extract of Greek extra virgin olive oil. Other oil extracts showed lower antimicrobial efficacy compared to extra virgin olive oil extracts. The unbuffered Spanish extra virgin olive oil extract inhibited most *Arcobacter*-like species after only 5 min of exposure. Strain *Al. thereius* LMG 24488 was able to survive 60 min exposure in the extracts with the highest antimicrobial content. T he mixed olive oil extract fully inhibited *Arcobacter*-like species strains for 30–180 min exposure. Higher concentrations of olive oil extracts usually resulted in increased biofilm formation. However, biofilm formation was inhibited in the highest concentration of unbuffered oil extracts tested. Presumably, the stress conditions for the planktonic cells were increased and they could not survive in this form and immediately concentrated into a more resistant form of biofilm.

Biofilm formation is an important virulence factor and represents a potential hazard as well as a significant risk to human health. There is no single mechanism for biofilm formation, but cellular stress appears to play a role in many cases. A full understanding of this process may help in the development of new antimicrobial agents capable of inhibiting its formation.

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7 List of Student´s Published Works

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  https://doi.org/10.3390/molecules27144509. **(IF 4.92)**

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  p. 1–12. https://doi.org/10.3390/ antibiotics11010087. **(IF 5.22)**

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