

IN VITRO ACTIVITY OF SPICE EXTRACTS AGAINST ARCOBACTER SPP. AND INFLUENCE ON THEIR BIOFILM FORMATION

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ABSTRACT

Several plants are considered to be new resources of substances that can be a potential alternative to combat particularly resistant microorganisms. *Arcobacter* spp. are dangerous bacteria with pathogenic potential and biofilm formation ability. The aim of this study was to evaluate the antimicrobial activity of six spice extracts against several *Arcobacter*-strains, including much less literally described strains as well. Among the evaluated spices, significant antibacterial activity of many samples was showed. The clove MetOH/DMSO (extraction in methanol/final dissolution in dimethylsulfoxide) extract had a MIC \geq 20 mg/mL, nutmeg MetOH/DMSO extract had MIC 10-20 mg/mL, and the highest values were recorded in star anise extract (MIC \geq 20 mg/mL) after 24 hours of exposure. However, arcobacters were highly resistant to extracts from juniper, nutmeg and anise. Furthermore, the effect of extracts on biofilm formation was monitored using Christensen method. The tested extracts were found to have a dual effect on biofilm formation. The elimination of biofilm formation was observed, however also its stimulation, depending on the type of extract and the bacterial strain. The results indicate a particularly interesting antimicrobial effect of cloves and cinnamon extract (inhibition zones up to 30 mm) against *Arcobacter* strains. The study shows a promising effect of tested extracts on biofilm formation for the first time.

Keywords: *Arcobacter* spp., biofilm, biofilm formation, natural extracts, biofilm inhibition

INTRODUCTION

Plants and their secondary metabolites are often used for their effects in the food industry or in healing. This is how spices, such as those known from ancient times, are used; not only for their aromatic ingredients but also for antimicrobial effects (Cowan, 1999). Many plants contain antimicrobial agents that inhibit the growth of food pathogens (Gyawali and Ibrahim, 2014; Mostafa et al., 2018; Tiwari et al., 2009). In general, gram-negative bacteria are less sensitive to antimicrobial agents than gram-positive, but this is not always the case (Tajkarimi et al., 2010).

The influence of natural extracts (eg. extracts from cinnamon, cloves, cilantro, thyme, star anise, garlic, onion, ginger, black pepper, etc.) on various bacteria has been observed in the past (Cervenka et al., 2006; Wang et al., 2012) and many have been found to have an antimicrobial effect. Microorganisms often occur in two forms, in the form of planktonic cells and microbial biofilms. The existence of microorganisms in a biofilm provides a wide range of benefits (resistance to antimicrobial agents, to drying, easier exchange of genetic material, nutrient transfer between cells, etc.). Biofilms are structured cell cultures adhered to the surface of a substrate and covered with extracellular matrix (Chmielewski and Frank, 2003; Flemming and Wingender, 2010). Biofilm formation itself is influenced by genetic and many other factors (eg. surface properties, pH, amount of available nutrients, temperature, specificity of the bacterial strain, etc.) (Borucki et al., 2003; Donlan, 2002; Chmielewski and Frank, 2003).

Among the bacteria occurring in both planktonic and biofilm form, there are also bacteria of the *Arcobacter* genus (Collado and Figueras, 2011; Hrušková et al., 2013; Šilhová et al., 2015). *Arcobacter* are bacteria characterized not only by a broad distribution of occurrence, but also by resistance to various antimicrobial agents and influences (Collado and Figueras, 2011; Kučerová et al., 2017; Shah et al., 2011; Šilha et al., 2013; Šilha et al., 2014; Šilha et al., 2015; Šilha et al., 2016; Šilha et al., 2017; Šilha et al., 2018). *Arcobacter* genus belongs to the family of *Campylobacteraceae* and includes both pathogenic (*A. butzleri*, *A. cryaerophilus*, *A. skirrowii* and *A. thereius*), as well as commonly occurring species (Perez-Cataluna et al., 2018; Van den Abeele et al., 2014).

The aim of this study was to evaluate the inhibitory effects of natural ethanol and methanol extracts (cloves, star anise, anise, cinnamon, juniper and nutmeg

extract) dissolved in dimethylsulfoxide (DMSO) but also in ethanol against *Arcobacter* spp. There is a number of factors that can affect not only the growth of arcobacters but also their biofilm formation. The influence of natural extracts on the formation of *Arcobacter* biofilms is not well documented in the literature and this study includes new finding in this area. Among these are the antimicrobial effects of natural substances such as extracts from herbs and spices, essential oils, etc. Biofilm formation was observed by the Christensen method in selected *Arcobacter* strains, and the effect of selected natural extracts on biofilm formation was monitored. Furthermore, the tested extracts were found to have a dual effect on biofilm formation of *Arcobacter* spp. Elimination of biofilm formation was observed as well as its stimulation depending on the type of extract and the bacterial strain (0.02-20 mg/mL). The study also included significantly less known species of arcobacters (*A. defluvii*, *A. thereius*, *A. lanthieri*), which have only been described in recent years.

MATERIAL AND METHODS

Plant materials and extraction procedure

The selected dried spices *Syzygium aromaticum* (clove, bud, origin – Madagascar), *Illicium verum* (star anise, fruit, origin – China), *Myristica fragrans* (nutmeg, seed, origin – Indonesia), *Pimpinella anisum* (anise, seed, origin – Spain), *Juniperus communis* L. (juniper, fruit, origin – Macedonia), and *Cinnamomum zeylanicum* (cinnamon, bark, origin – Vietnam) were bought from local markets in the Czech Republic (distributor – Vitana a.s., Czech Republic). After crushing the plant parts, methanol p.a. (MetOH; Lach-Ner, Czech Republic) or ethanol p.a. (EtOH; Lach-Ner, Czech Republic) (1:2 w/v) was used for the extraction of all spices. Extractions were conducted at room temperature for 4 days in the dark. After filtration, the filtrates were concentrated by evaporation to dryness. Dry extracts were dissolved in 20% dimethylsulfoxide (DMSO; Penta, Prague, Czech Republic) or 96% ethanol to a final concentration of 100 mg/mL (stock solution). The microbial sterility of the prepared extracts was verified before each testing.

Antimicrobial testing

The microorganisms used in this study were as follows – *A. butzleri* CCUG 30484, *A. butzleri* UPa 2013/8 (strain isolated from waste water treatment), *A. cryaerophilus* CCM 7050, *A. cryaerophilus* UPa 2013/13 (strain isolated from waste water treatment), *A. butzleri* LMG 10828, *A. skirrovii* LMG 6621, *A. defluvii* LMG 25694, *A. thereius* LMG 24488, and *A. lanthieri* LMG 28517. Strains were purchased from the Czech Collection of Microorganisms (CCM, Brno, Czech Republic), Culture Collection University of Göteborg (CCUG, Göteborg, Sweden), Belgian Co-ordinated Collections of Microorganisms (LMG, Ghent, Belgium), and isolated at the University of Pardubice (UPa, Pardubice, Czech Republic). Cultures were grown on Mueller–Hinton agar (MHA, Oxoid Ltd., Basingstone, United Kingdom) before antimicrobial testing. Cells were harvested and suspended in physiological saline to a 0.5 McFarland scale (ca 1.5×10^8 CFU/mL).

For antibacterial testing, the agar disc diffusion method was used. Plates containing Mueller–Hinton agar were spread with bacterial suspension adjusted to 1.5×10^8 CFU/mL. Filter paper discs (6 mm diameter, Oxoid Ltd.) were placed onto inoculated agar surfaces and impregnated with 8 μ l of stock solution. Simultaneously, pure DMSO and EtOH were used as a negative control while ampicillin, ciprofloxacin, clindamycin, erythromycin, and tetracycline discs (Oxoid Ltd.) were used as a positive control. The plates were cultivated for 48 h at 30 °C under aerobic conditions. Inhibition zones were measured using a Bacmed 6iG2 automatized reader (Aspiag, Czech Republic). Experiments were performed in triplicates and the resulting activities (mm) were expressed as a mean of inhibition zones (mm) with standard deviation.

Determination the survival of strains in extract presence

The effect of various concentrations of selected MeOH/DMSO extracts (clove, star anise, nutmeg) on planktonic cells was monitored. Briefly, the twofold dilutions of extracts were prepared in BHI broth (Oxoid Ltd.) to obtain a final concentration ranging from 20 to 0.02 mg/mL in the wells after the addition of 20 μ l of the freshly diluted tested cell culture containing 10^8 CFU/mL. Before and after incubation at 30 °C for 24 h under aerobic conditions, the absorbance was measured (TECAN Infinite M 200, Tecan Trading AG; Switzerland). At the same time, the culture after incubation was spread onto cultivation medium to determine whether the strain was culturable.

Effect of natural extracts on biofilm formation

The clove, star anise, and nutmeg MeOH/DMSO extracts were tested to evaluate their effect on biofilm formation. The effect of different concentrations (0.02–20 mg/mL) of the extracts on biofilm formation was monitored in 96-well polystyrene flat-bottomed microtiter plates (SPL Live Sciences Co., Ltd., Korea) as previously described (Christensen et al., 1985) with modifications. Briefly, the twofold dilutions of extracts were prepared in BHI broth to obtain a final concentration ranging from 20 to 0.02 mg/mL in the wells after the addition of 20 μ l of the freshly diluted tested cell culture containing 10^8 CFU/mL. After incubation at 30 °C for 24 h under aerobic conditions, the microtiter plate was repeatedly washed (sterile distilled water) and dried. Fixation of the biofilm was performed with 2% sodium acetate (15 min), and attached cells were stained with 100 μ l of 1% crystal violet. After 15 min of staining, the plate was repeatedly washed and dried. Then the biofilm-associated crystal violet was solubilized with 96% ethanol, and the absorbance of the solution was measured in a new plate at 595 nm (TECAN Infinite M 200, Tecan Trading AG; Switzerland).

In addition, the effect of extract particles was monitored as an increasing in the final absorbance of the crystal violet after the staining process (Table 2). The experiments were performed as above, but without added bacterial cells. The effect of the extract components on the final absorbance value was eliminated by reducing the absorbance contribution of the extract (Fig 1-3).

There were 8 replicate wells in each experiment, experiments were independently repeated 3 times. The obtained values were statistically evaluated using Excel 2010 (Microsoft, USA) and Statistica 12 (StatSoft, USA). Extreme values were tested by the Dean-Dixon test, and any outlier values were excluded with 95% probability. The mean and standard deviation were determined from the remaining values. A possible source of mistakes was also considered, which is the effect of insufficient dye washing or the influence of the components of the extracts used, resulting in an increase in absorbance. Similarly, absorbance values that were too high compared to other measured values were excluded.

RESULTS AND DISCUSSION

Antibacterial effect of natural extracts

The effect of natural extracts and essential oils against *Arcobacter* spp. and *Campylobacter* spp. has been studied in several earlier studies (Cervenka et al., 2006; Irkin et al., 2011; Thanissery et al., 2014). However, most of the earlier studies only deal with the most well-known and most described species, *A. butzleri* and *A. cryaerophilus*. In our study, some of the recently described

Arcobacter species – *A. defluvii*, *A. lanthieri* – have also been included in the study, and also *A. thereius*, whose pathogenic potential has already been described (Van den Abeele et al., 2014).

The results of the antibacterial activity of the tested extracts are shown in Table 1. Each sample was extracted in two portions, ethanol and methanol. The extracts obtained were dissolved in DMSO (no inhibitory effect on the test bacteria) and ethanol (inhibitory effect of pure solvent 8-10 mm depending on the strain) at a final concentration of 100 mg/mL. There was no significant difference between the antibacterial effects of ethanol and methanol extracts.

It is generally apparent from the results that the extracts finally dissolved in ethanol showed a logically higher antibacterial activity compared to those dissolved in DMSO. Very interesting antibacterial effects against *Arcobacter* spp. were recorded with cinnamon (inhibition zones: 7-16 mm – extracts in DMSO, and 10-32 mm – extracts in EtOH) and cloves extracts (inhibition zones: 8-18 mm – extracts in DMSO, and 13-21 mm – extracts in EtOH). The largest inhibition zones (up to 30 mm, and 28.5 mm) were found for *A. cryaerophilus* CCM 7050, and *A. cryaerophilus* UPa 2013/13 in case of EtOH/EtOH cinnamon extract.

On the other hand, the lowest antimicrobial activity against arcobacters was found for the juniper (inhibition zones: 6-7 mm – extracts in DMSO, and 10-14 mm – extracts in EtOH) and nutmeg extracts (inhibition zone: 6-8 mm – extracts in DMSO, and 9-13.5 mm – extracts in EtOH). Anise extracts were slightly antibacterially active against most *Arcobacter* strains, but its highest inhibitory effect (inhibition zone up to 14 mm) was observed against *A. skirrovii* LMG 6621. Similarly, the star anise extract was slightly antibacterially effective against most tested *Arcobacter*-strains. Its highest antimicrobial effect was against *A. cryaerophilus* CCM 7050, whereas the *A. lanthieri* LMG 28517 strain was comparatively resistant to this extract.

Among the traditional strains of the *Arcobacter* genus (*A. butzleri*, *A. cryaerophilus*) there was a difference in susceptibility to the tested extracts. However, it was not possible to say whether the strains from collections were more or less susceptible than the strains isolated from the environment.

Overall, the strain *A. lanthieri* LMG 28517 was the most resistant of all the strains included in this study to the tested natural extracts. The highest zone of inhibition was found for extracts dissolved in DMSO, even in extracts which had a significant antibacterial effect on other *Arcobacter*-strains.

The antimicrobial activity of 8 plant essential oils against *A. butzleri* strains was previously tested (Irkin et al., 2011). Oil obtained from cloves was able to inhibit the growth of all arcobacters tested. The sensitivity of campylobacters has also been described for essential oils of rosemary, thyme, cloves and oranges (Thanissery et al., 2014). To the best of our knowledge, there is no information available in the literature on testing the antimicrobial effect of star anise and nutmeg against arcobacters. However, the antimicrobial activity of the star anise extract has been confirmed previously in case of other microorganisms (De et al., 2002). Similarly, for nutmeg extract, its antimicrobial efficacy has also been confirmed in previous studies (Gupta et al., 2013; Takikawa et al., 2002).

The previous study describes the effect of 17 methanol and chloroform extracts from some herbs and spices against three *Arcobacter* collection strains (Cervenka et al., 2006). Methanol extracts were rated as more antibacterially active, with the highest activity in rosemary, allspice, thyme, camomile, cinnamon, and others. According to our results, the inhibitory effect of cinnamon and clove was particularly high. However, due to the different testing procedure and the strains used, these results are not entirely comparable.

Survival of planktonic cells in the presence of the natural extracts

The viability of the strains after 24 hours of culture in the presence of selected MeOH/DMSO extracts was evaluated by increasing the absorbance after cultivation and at the same time by cultivating on agar media. With the clove extract, almost all strains were found to survive at all concentrations (MIC \geq 20 mg/mL). However, the *A. cryaerophilus* UPa 2013/13 and *A. skirrovii* LMG 6621 strains did not survive the 24-h exposure at a concentration of 20 mg/mL (MIC 20 mg/mL). The nutmeg extract was inhibitory to *A. butzleri* CCUG 30484 (MIC 20 mg/mL), *A. butzleri* LMG 10828 (MIC 20 mg/mL), *A. cryaerophilus* CCM 7050 (MIC 20 mg/mL), *A. skirrovii* LMG 6621 (MIC 10 mg/mL) and *A. thereius* LMG 24488 (MIC 10 mg/mL). However, all of the strains tested survived the 24-h exposure at all the tested concentrations of the star anise extract.

Biofilm formation in the natural extracts presence

Due to biofilm resistance, the higher resistance of microorganisms in a biofilm is often discussed, leading to antimicrobial therapy failure. It is estimated that 65-80 % of all resistant infections are caused by bacteria in the form of a biofilm (Dautle et al., 2003; Flemming and Wingender, 2010). Biofilm formation is a significant risk factor for the food industry, as well as other areas. The ability to produce a biofilm in *Arcobacter* spp. has already been described in the literature (Collado and Figueras, 2011), but this information is still missing for the species described in the last 10 years. According to our results, *A. defluvii*, *A. lanthieri* and *A. thereius* are species capable of biofilm formation and according to our designation of the tested strains, they belong to the moderate producers of

biofilms. Initial biofilm activity of the studied strains (at concentration of 0 mg/mL) was at different levels. The highest biofilm activity was observed for *A. butzleri* UPa 2013/8 ($A=0.180-0.200$). In contrast, *A. butzleri* CCUG 30484 and *A. cryaerophilus* UPa 2013/13 strains were rated as poor/nonbiofilm producers ($A<0.120$). Other *Arcobacter* strains were rated as moderate biofilm producers ($A = 0.120-0.140$). Furthermore, the biofilm activity of arcobacters in the presence of selected extracts of spices was studied.

$A_{cut-off score}$ (A_{COS}) was determined to evaluate the biofilm production results. The value of $A_{COS-BHI}=0.112$ represents the final absorbance after coloring

the remainder of the uninoculated BHI broth, and it is shown as a blank horizontal line in Fig 1-3. Furthermore, the A_{COS-EX} for the individual tested extract concentrations was determined (Table 2) and this value was deducted from the final absorbance (A), as it represents the effect of the extract matrix and has no relation to biofilm formation. Bacterial strains whose $A \leq A_{COS-BHI}$ were considered biofilm-negative and strains with $A > A_{COS-BHI}$ were considered biofilm-positive.

Table 1 Antimicrobial effect of natural extracts of spices, used dilution solvent, and standard antibiotics (mean inhibition zones in mm, including disc 6 mm in diameter \pm standard deviation), n=3

	Extraction solvent	Dilution solvent	<i>A. butzleri</i>			<i>A. cryaerophilus</i>		<i>A. skirrowii</i> LMG 6621	<i>A. thereius</i> LMG 24488	<i>A. lanthieri</i> LMG 28517	<i>A. defluvi</i> LMG 25694
			CCUG 30484	LMG 10828	UPa 2013/8	CCM 7050	UPa 2013/13				
Cinnamon extract	EtOH	DMSO	15.5±0.5	8±0.0	9±0.5	13±0.5	12±0.5	10±0.5	10±0.5	7.5±0.5	7±0.0
	EtOH	EtOH	17.5±0.0	12±0.0	14.5±0.5	30±1.5	28.5±1.0	28±1.5	21.5±0.5	13±0.5	10.5±0.5
	MetOH	DMSO	14.5±0.0	8±0.5	9±0.5	13±0.5	10.5±0.5	11±0.5	11±0.5	8±0.0	7±0.0
	MetOH	EtOH	17.5±0.0	12.5±0.5	13.5±0.5	23±0.5	26.5±0.5	23±1.0	21.5±0.5	14±0.5	10.5±0.5
Clove extract	EtOH	DMSO	14.5±0.5	12±0.0	13.5±0.5	14±0.0	16±0.0	12±0.5	12±0.5	8±0.0	14.5±0.5
	EtOH	EtOH	14.5±0.0	13.5±0.0	14±0.0	14.5	18±0.5	20±0.0	14.5±0.0	14.5±0.5	17.5±0.0
	MetOH	DMSO	16.0±0.0	12.5±0.5	14.5±0.5	15±0.5	18±0.0	13±0.5	10±0.0	8±0.0	17±0.5
	MetOH	EtOH	15.5±0.0	15±0.0	14.5±0.0	17±0.0	20±0.5	20.5±0.0	15±0.0	11.5±0.5	19±0.0
Star anise extract	EtOH	DMSO	8±0.5	8±0.5	8±0.5	8±0.5	9±0.5	8±0.5	8±0.0	7±0.0	8±0.5
	EtOH	EtOH	13±0.5	13±0.5	13.5±0.5	16±0.5	13±0.5	13.5±0.5	14±0.5	12.5±0.5	13±0.0
	MetOH	DMSO	8±0.5	8±0.0	8±0.5	8±0.0	8.5±0.5	8±0.5	8±0.5	7.5±0.5	8±0.5
	MetOH	EtOH	12.5±0.5	13±0.5	14±0.0	15±0.5	13±0.5	13±0.5	14±0.5	12±0.5	13±0.5
Anise extract	EtOH	DMSO	6.5±0.0	6.5±0.5	7±0.5	7±0.0	8±0.5	8.5±0.5	7±0.0	7±0.5	7±0.5
	EtOH	EtOH	11±0.5	12±0.5	13±0.0	12.5±0.0	10.5±0.5	13.5±0.5	13.5±0.0	11±0.5	11.5±0.0
	MetOH	DMSO	n.i.	6.5±0.5	6.5±0.5	7±0.5	8±0.5	8±0.5	7±0.5	7±0.5	7±0.5
	MetOH	EtOH	11.5±0.5	12±0.5	13.5±0.5	13±0.5	11±0.5	14±0.5	13.5±0.5	12±0.5	11±0.5
Nutmeg extract	EtOH	DMSO	n.i.	n.i.	n.i.	n.i.	8±0.5	7±0.5	7±0.5	n.i.	n.i.
	EtOH	EtOH	10.5±0.5	10.5±0.5	12.5±0.5	10±0.5	12±0.5	12±0.5	13±0.5	10.5±0.5	11.5±0.5
	MetOH	DMSO	n.i.	n.i.	n.i.	n.i.	7±0.0	7±0.5	7±0.5	n.i.	n.i.
	MetOH	EtOH	9±0.5	12±0.5	12±0.5	10.5±0.5	12±0.5	12±0.5	13.5±0.0	12.5±0.0	12.5±0.0
Juniper extract	EtOH	DMSO	n.i.	n.i.	n.i.	n.i.	n.i.	7.5±0.5	n.i.	n.i.	n.i.
	EtOH	EtOH	10.5±0.5	10±0.5	11±0.5	13.5±0.0	10.5±0.5	13±0.5	13.5±0.5	10±0.5	10.5±0.5
	MetOH	DMSO	n.i.	n.i.	n.i.	n.i.	n.i.	7±0.5	n.i.	n.i.	n.i.
	MetOH	EtOH	11.5±0.5	12±0.5	11±0.5	12±0.5	11.5±0.5	12±0.5	12.5±0.5	10±0.5	12±0.5
EtOH		8.5±0.5	9.0±0.5	9.0±0.5	9.0±0.5	9.5±0.5	10.0±0.5	9.5±0.5	8.5±0.5	9.5±0.5	
DMSO		n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	
CIP 5 µg		48.0±0.0	56.0±0.0	42.0±0.0	32.0±0.0	40.0±0.0	42.0±0.0	40.0±0.0	44.0±0.0	42.0±0.0	
E 15 µg		40.0±0.0	31.0±0.0	25.0±0.0	25.0±0.0	25.0±0.0	25.0±0.0	30.0±0.0	25.0±0.0	25.0±0.0	
TE 30 µg		29.0±0.0	30.0±0.0	25.0±0.0	25.0±0.0	27.0±0.0	25.0±0.0	44.0±0.0	18.0±0.0	25.0±0.0	
AMP 30 µg		n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	
DA 2 µg		n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	

Legend: EtOH – ethanol, MetOH – methanol, DMSO – dimethylsulfoxide, AMP – ampicillin, CIP – ciprofloxacin, DA – clindamycin, E – erythromycin, TE – tetracycline, n.i. – not inhibited

Table 2 Measured and calculated *cut-off scores* ($A=595$ nm) of natural extracts

Concentration (mg/mL)		0.020	0.039	0.078	0.156	0.313	0.625	1.125	2.5	5	10	20
Clove extract	A_M	0.126	0.130	0.124	0.131	0.131	0.129	0.133	0.136	0.151	0.139	0.176
	A_{COS-EX}	0.014	0.018	0.012	0.019	0.019	0.017	0.021	0.024	0.029	0.027	0.064
Star anise extract	A_M	0.113	0.116	0.117	0.128	0.129	0.125	0.122	0.120	0.123	0.134	0.213
	A_{COS-EX}	0.001	0.004	0.005	0.017	0.018	0.013	0.011	0.008	0.011	0.022	0.101
Nutmeg extract	A_M	0.124	0.129	0.130	0.130	0.131	0.136	0.132	0.134	0.132	0.141	0.267
	A_{COS-EX}	0.012	0.017	0.018	0.019	0.020	0.024	0.020	0.022	0.020	0.029	0.155

Legend: A_M – median of measured absorbance of extracts in BHI broth, A_{COS-EX} – absorbance of extract (the increase in the value due to the content of the extract components)

To determine the effect of natural extracts on biofilm formation, methanol extracts from cloves, nutmeg and star anise were chosen, finally dissolved in DMSO (MetOH/DMSO). The dependence of biofilm formation in the presence of natural extracts is shown in Fig 1-3. The tested extracts were been found to

have a dual effect on biofilm formation. On the one hand, an elimination of biofilm formation was observed, but also its stimulation, dependent on the type of extract and the given bacterial strain.

Based on Fig 1-3, it can be stated that the tested natural extracts exhibited a similar effect on biofilm formation in all *Arcobacter* strains (similar trend), apart from some exceptions. Biofilm formation generally decreased with increasing concentration of the extract. A rapid decline in biofilm formation was observed at the highest extract concentration of 20 mg/mL, for all tested strains and extracts. The biofilm formation of the studied arcobacters was thus successfully inhibited by higher concentrations of the extracts. The clove extract was rated as the most effective in terms of the decline in biofilm formation of actually all *Arcobacter* spp. (Fig 1). Even at the lowest extract concentration (0.02 mg/mL), biofilm formation declined (the decline in absorbance from 0.189 to 0.127) rapidly in case of *A. butzleri* UPa 2013/8 strain. In *A. butzleri* CCUG 30484, *A. butzleri* LMG 10828 and *A. thereius* LMG 24488,

complete biofilm inhibition occurred at this concentration. *A. skirrovii* LMG 6621 was evaluated as the resistant strain, with complete inhibition of biofilm formation at the concentration of 5 mg/mL of clove extract. The MBIC of clove extract was found even at the concentration of 20 mg/mL for the *A. defluvii* LMG 25694 strain. When comparing the strains of *A. cryaerophilus*, it was found that the collection strain was more biofilm-active and also forms biofilm in a medium with higher concentrations of the extract than the isolate of the same species. Further, minimal biofilm inhibitory concentration (MBIC) determination and expression was performed, and the results are presented in Table 3. The MBIC is the minimal concentration of the natural compounds at which the biofilm formation was reduced to $A_{595} < 0.112$.

Table 3 Minimal biofilm inhibitory concentration (MBIC; mg/mL) of selected MetOH/DMSO natural extracts, n=3

	<i>A. butzleri</i>			<i>A. cryaerophilus</i>		<i>A. skirrovii</i>	<i>A. thereius</i>	<i>A. lanthieri</i>	<i>A. defluvii</i>
	CCUG 30484	LMG 10828	UPa 2013/8	CCM 7050	UPa 2013/13	LMG 6621	LMG 24488	LMG 28517	LMG 25694
Clove extract	0.02	0.02	1.13	0.62	0.32	5.0	0.02	0.16	20.0
Nutmeg extract	0.04	0.02	1.13	0.16	0.08	5.0	0.08	5.0	2.5
Star anise extract	0.16	0.16	20.0	0.16	0.16	5.0	20.0	10.0	20.0

Legend: MetOH/DMSO – extraction in methanol/final dissolution in dimethylsulfoxide

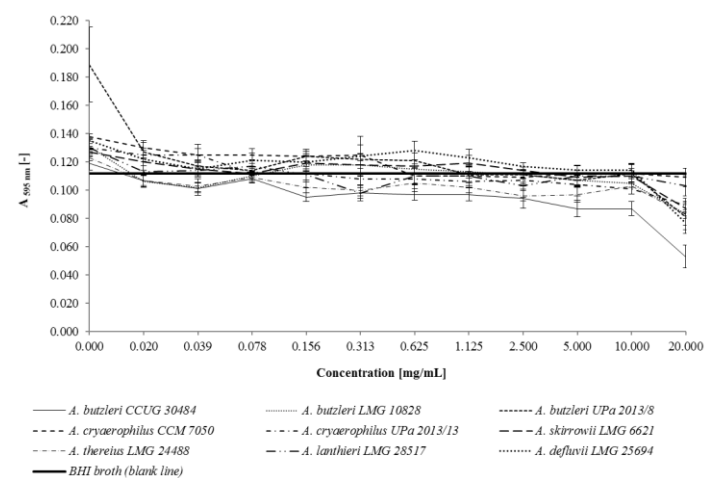


Figure 1 Effect of clove extract on biofilm formation of *Arcobacter* spp. (mean of measured data ± standard deviation), n=3. The horizontal line represents the influence of BHI broth (values under horizontal line – biofilm-negative; values above line – biofilm-positive)

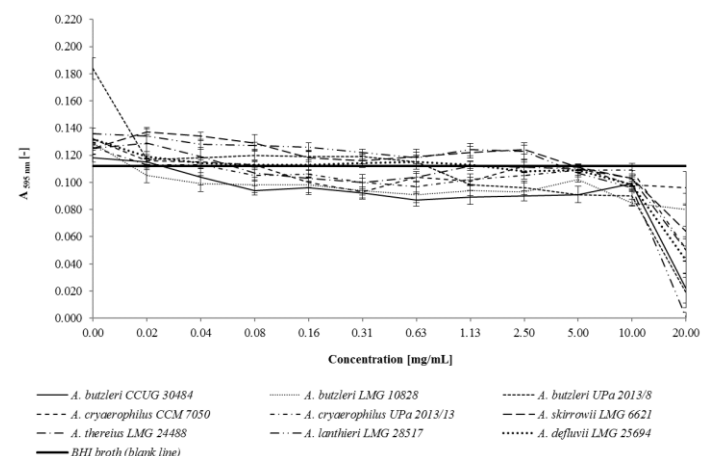


Figure 2 Effect of nutmeg extract on biofilm formation of *Arcobacter* spp. (mean of measured data ± standard deviation), n=3. The horizontal line represents the influence of BHI broth (values under horizontal line – biofilm-negative; values above line – biofilm-positive)

The nutmeg extract eliminated the formation of a biofilm in *A. butzleri* LMG 10828 (Fig 2) at a low concentration of 0.02 mg/mL, while in case of the collection strain (*A. butzleri* CCUG 30484) the MBIC was observed at a concentration of nutmeg extract of 0.04 mg/mL. Overall, *A. butzleri* collection

strains were evaluated as the easiest to suppress biofilm formation with this extract. Conversely, with *A. skirrovii*, at a concentration of 0.02 mg/mL, the biofilm formation was increased from an initial value of 0.125 to 0.137 with a following gradual decrease and the MBIC was found to be 5 mg/mL. A similar, yet milder effect was also observed in *A. thereius*.

Overall, star anise extract had the least antibacterial and antibiofilm effects against arcobacters. There was even a sharp increase in biofilm formation in the presence of lower concentrations of the extract, with *A. lanthieri* LMG 28517 (initial value of A without the effect of extract was 0.129; A increased to 0.174 at a concentration of 0.02 mg/mL) and *A. butzleri* CCUG 30484 (initial value of A without the effect of extract was 0.120; A increased to 0.128 at a concentration of 0.02 mg/mL). *A. butzleri* UPa 2013/8 retained a relatively high biofilm activity even at concentrations of star anise extract of about 1 mg/mL (Fig 3). In some strains, inhibition of biofilm formation was observed at an extract concentration of 0.156 mg/mL, but in *A. butzleri* UPa 2013/8, *A. thereius* LMG 24488 and *A. defluvii* LMG 25694, biofilm formation was completely inhibited at a concentration of 20 mg/mL.

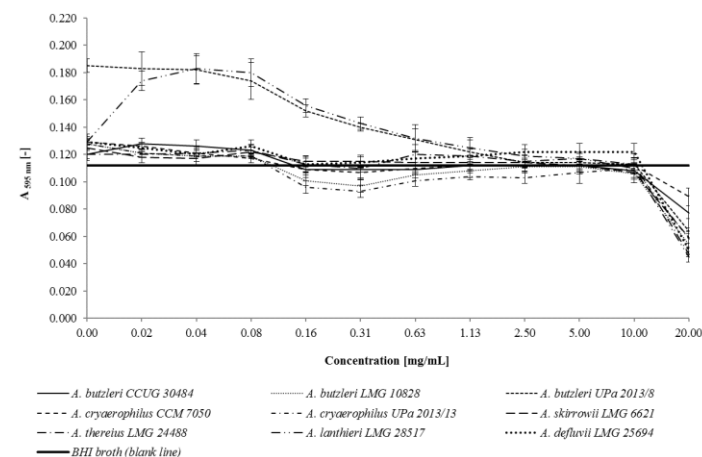


Figure 3 Effect of star anise extract on biofilm formation of *Arcobacter* spp. (mean of measured data ± standard deviation), n=3. The horizontal line represents the influence of BHI broth (values under horizontal line – biofilm-negative; values above line – biofilm-positive)

The influence of natural extracts on the formation of *Arcobacter* biofilms is not well documented in the literature. In our study, the effect of natural substances has been shown to be double effect. Some spices extracts (especially at lower concentration) stimulated the biofilm formation, on the other hand, other extracts eliminated biofilm formation. The stimulation of biofilm formation has been shown in particular in the case of star anise extract. The increase in biofilm formation at lower concentrations of star anise extract could be explained by the lower antimicrobial effect of this extract. Cells were prevented by increased biofilm formation at lower concentrations, however, this led to complete suppression of biofilm activity with increasing concentration. However, the effects of phenolic compounds on *Pseudomonas aeruginosa* biofilm have been described in the past. With these pure forms of phenolic compounds, the stimulation of biofilm formation was observed and inhibition only occurred at

very high concentrations (Plyuta et al., 2013). Similarly, the inhibition of biofilm formation in *Klebsiella pneumoniae* was monitored with several natural substances (berberin, reserpin, chitosan, curcumin, eugenol and linoleic acid). In this work, the inhibition of biofilm activity and MBIC were determined in the range of 0.0156-0.25 mg/mL (Magesh et al., 2013). Weak antibiofilm activity was described for garlic extract with selected bacteria (Mohsenipour and Hassanshahian, 2015).

CONCLUSION

Natural substances isolated from plant matrices are currently required for their positive effects in the fight against microorganisms. We have presented inhibitory effect of clove, star anise, nutmeg, anise, juniper and cinnamon extracts against bacteria of the genus *Arcobacter*. This genus includes many species isolated from various environment, however, many studies deal with only a few of the best known species (*A. butzleri* and *A. cryaerophilus*). Moreover, our study includes other much less known species of arcobacters and thus bring new information to knowledge. The results show that clove and cinnamon extracts have the highest inhibitory effect from all extracts differently prepared and diluted in various solvents (DMSO, ethanol). Further, the present study demonstrated the effect of clove, nutmeg and star anise extracts on biofilm formation of nine *Arcobacter* strains. In general, the influence of natural extracts on the biofilm formation is not well documented in the literature and this study includes new finding in this area. To the best of our knowledge, this is the first study reporting the antibiofilm activity of the spice extracts against *Arcobacter* spp. The tested extracts were found to have a dual effect on biofilm formation of *Arcobacter* spp. On the one hand, the elimination of biofilm formation was observed (especially in higher concentration of tested extracts), but also its stimulation (e.g. in case of star anise extract), depending on the type of extract and the bacterial strain. The results of the study demonstrate that many spices inhibit the growth of potential pathogenic bacteria from the genus *Arcobacter*, either in planktonic or in biofilm phenotype, however, another testing is needed.

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