INVESTIGATION AND DISRUPTION OF BACTERIAL BIOFILMS IN THE VETERINARY MEDICINE

STUDY REPORT

THE SUMMARY AND STUDY REPORT CONTAINS CONFIDENTIAL INFORMATION, PLEASE HANDLE PRIVATELY, AS FAR AS POSSIBLE.

1. DETERMINATION OF THE PREVALENCE OF BIOFILM PRODUCTION IN *P. AERUGINOSA*, *S. PSEUDINTERMEDIUS* ISOLATED FROM CANINE EAR CANAL INFECTIONS, AND *S. AUREUS* STRAINS ISOLATED FROM BOVINE SUBCLINICAL MASTITIS.

The first goal of our investigations was to determine the intensity of biofilm formation by each bacterial strain. *S. pseudintermedius* strains were tested on Congo Red Agar (CRA) and microtiter-plate test using crystal violet staining, while *P. aeruginosa* and *S. aureus* strains were examined by crystal violet staining alone. In our study, 80 *S. pseudintermedius*, 73 *P. aeruginosa* and 4 *S. aureus* strains were tested. Three ATCC reference strains: two biofilm-producer (*P. aeruginosa* ATCC 27853 and *S. aureus* ATCC 35556), and a non-biofilm producer (*S. epidermidis* ATCC 12228) served as positive and negative controls. Intensity of biofilm formation was first evaluated by crystal violet staining on 96-well microtiter plates. To form biofilms, strains were incubated for 24 hours in Mueller-Hinton broth at 37ºC. The volume of the produced biofilm biomass was detected by the application of 0.1% crystal violet solution, then absorbance values were measured by spectrophotometer at 595 nm. CRA method was performed to examine colony morphology after 24 hours of incubation at 37ºC. Biofilm formation of the strains was determined macroscopically by the colour of the colonies: strains forming black and dark gray colonies were considered as biofilm producers, while non-biofilm producers were red in colour.

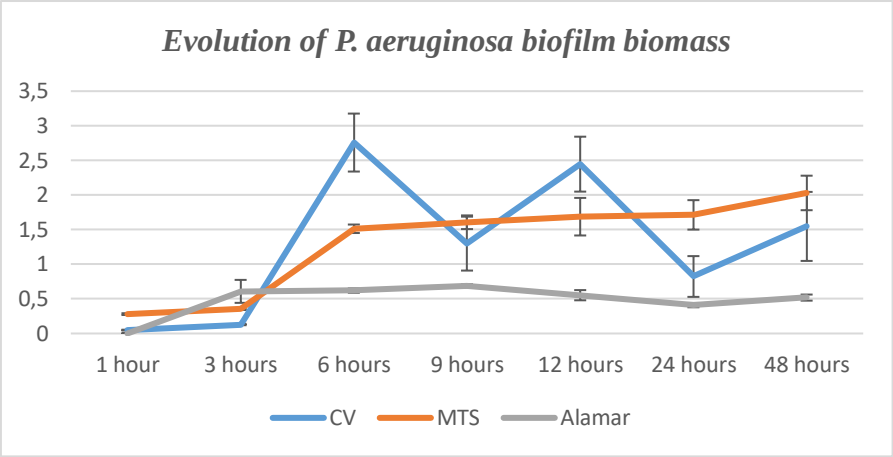
According to the results, bacterial strains were classified into 4 groups: strong, moderate, weak and non-biofilm producers. Among the 73 tested strains of *P. aeruginosa*, 6 (8%) were classified as non-biofilm producers, 19 (26%) were weak, 24 (33%) were moderate, and 14 (19%) strains were strong biofilm producers tested by crystal violet staining. All 4 *S. aureus* strains examined in our study, were moderate biofilm producers. In the case of *S. pseudintermedius* strains tested by CRA method, 10 (12,5%) strains which formed red colonies, were classified as non-biofilm producers, while the other 70 (88,5%) strains, which formed

black or dark gray colonies, were classified as biofilm producers. Our experiments suggest that crystal violet staining is not a reliable method to examine biofilm formation in *S. pseudintermedius*, as higher intensity of biofilm formation was detected by the ATCC- strain than the ATCC+ strain in this case.

Investigating the evolution of bacterial biofilms over time

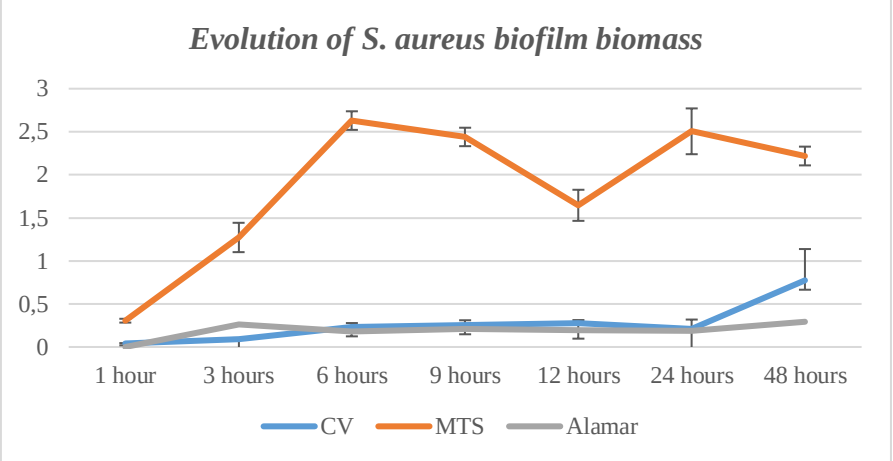
One of our primary goals was to investigate the evolution of biofilms of *S. aureus* and *P. aeruginosa* over time, in order to find out the most appropriate incubation periods usable in our following studies. Furthermore, this study was performed to compare the different assays used in the detection of biofilm formation. The evolution of biofilms produced by 1 *P. aeruginosa* and 1 *S. aureus* strains were tested. The *P. aeruginosa* strain was isolated from a dog suffering from otitis externa, while the *S. aureus* strain was isolated from bovine subclinical mastitis. Before the experiment, isolates were stored at -80°C using *Microbank*™ system (Pro-Lab Diagnostics, Richmond Hill, Canada). Initially, strains were inoculated into *Müller-Hinton* (MH) broth and incubated for 24 hours at 37°C. Thereafter, growth culture was added to MH broth to make a 1:100 dilution. Sterile 96-well microtiter plates were used to produce biofilms, where each well contained MH broth and the appropriate volume of diluted bacterial culture. Bacterial suspensions were incubated for 1, 3, 6, 9, 12, 24 and 48 hours at 37 °C. After the incubation periods, the quantity of the produced biofilm biomass was determined by crystal violet staining, while the amount of viable cells embedded in the biofilm was examined by MTS-formazan and Alamar Blue (resazurin) assays.

Biofilm biomass in investigated *P. aeruginosa* strain reached its maximum after 6 hours of incubation. Later, a periodical fluctuation was observable in the amount of biomass by crystal violet staining, a decrease between 6-9 hours, an increase between 9-12 hours, and later a significant decrease between 12-48 hours. Using MTS-formazan assay, viable cells embedded in the bacterial biofilm showed a remarkable increase between 3-6 hours. After 6 hours of incubation, there was a slowly increasing tendency in the amount of viable cells. Alamar Blue assay showed different results in the volume of viable cells, a significant increase between 1-3 hours, a slow increase between 3-9 hours and then a decreasing tendency from 9 to 24 hours of incubation. Between 24-48 hours, there was an additional slight increase.



Evolution of untreated *P. aeruginosa* biofilm biomass over time. The y-axis represents absorbance values which were measured spectrophotometrically, while the x-axis shows the different incubation periods when measurements were performed.

Biofilm biomass produced by the *S. aureus* strain poorly takes up crystal violet, this way the measured intensity is essentially less compared to the intensity of the biomass produced by *P. aeruginosa*. Crystal violet staining showed a slight increase between 1-24 hours and a notable increase from 24 hours to 48 hours of incubation. The amount of viable cells in the biofilm, detected by MTS-formazan assay, reached the maximum at 6 hours of incubation after a rapid increase between 1-6 hours. Between 6-12 hours, the number of viable cells decreased remarkably, but later it started to rise anew. According to the results of Alamar Blue assay, the amount of viable cells reached its maximum at 3 hours of incubation after rapid growth. Thereafter, a decrease was presented, and the volume of living cells was nearly constant from 6 to 24 hours. Between 24-48 hours, a notable increase was presented again.



Evolution of untreated *S. aureus* biofilm biomass over time. The y-axis represents absorbance values which were measured spectrophotometrically, while the x-axis shows the different incubation periods when measurements were performed.

According to our results, the MTS-formazan vital stain was the main method used in our studies.

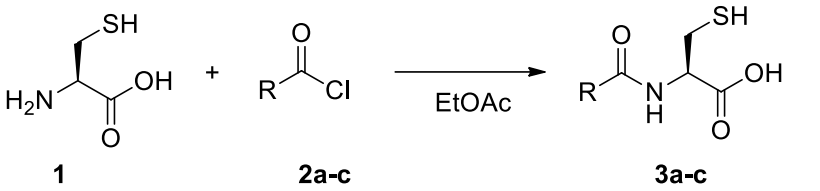
2. INVESTIGATION OF BIOFILM-ASSOCIATED GENES IN *P. AERUGINOSA* STRAINS

The main role of pellicle genes is to encode different enzymes participating in extracellular matrix synthesis during biofilm development. Detection of *pelA, pelC and pelE* genes in 24 *P. aeruginosa* strains was performed by PCR method. DNA of the isolates was extracted and purified using E.N.Z.A. bacterial DNA kit (OMEGA, Bio-Tek, Norcross, USA). The volume of the extracted DNA was measured by NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, USA) at 260 nm. PCR method was performed by MiniOpticon PCR device (BioRad, Hercules, USA) with the application of 5PRIME HotMasterMix (5PRIME GmbH, Hamburg, Germany). All of the three genes were detected, which proves the ability of biofilm formation of the examined *P. aeruginosa* isolates (see Figure 3 in the Appendix and Figure 4 confirmation with scanning electron microscopy).

3. INVESTIGATION OF SUBSTANCES ABLE TO DISTURB PREFORMED BIOFILMS OF *P. AERUGINOSA* AND *S. AUREUS* STRAINS

Substances investigated in this study

A large number of substances were investigated against *P. aeruginosa* and *S. aureus* biofilms: N-acetylcysteine, newly synthetised cysteine derivatives (N-monochloroacetyl-cysteine, N-dichloroacetyl-cysteine, N-benzoyl-cysteine), essential oils (geraniol, thyme oil, cinnamon oil), plant-derived extracts (mint, green tea, rosemary, water chestnut, baicalin), ethylenediaminetetraacetic acid (EDTA) and tryptophan. NAC derivatives were synthesized at the Department of Chemistry at the University of Veterinary Medicine, Budapest. During the synthesis, the primary aim was to use the least number of steps possible to prepare the substances. Therefore, cysteine (1) was directly N-acylated in ethyl acetate (EtOAc) with the appropriate acid chloride (2a-c) to produce phenyl, dichloro and monochloro substituted derivatives (3a-c). The crude products were purified by recrystallization.



1 **2a-c** **3a-c**

3a R = phenyl
3b R = -CH_2Cl
3c R = -$CHCl_2$

Bacterial strains and culture conditions

In this study, substances were tested against formed biofilms of 12 *P. aeruginosa* strains isolated from canine otitis externa, and 4 *S. aureus* strains isolated from bovine subclinical mastitis. Bacterial strains were stored at -80°C using *Microbank*^{TM} system (Pro-Lab Diagnostics, Richmond Hill, Canada), which permits rapid access to the stored organisms. Before the experiments, strains were inoculated into *Müller-Hinton* (MH) broth and incubated for 24 hours at 37°C. After the incubation period, bacterial suspensions were diluted until reaching 10^5 CFU/ml, then applied on 96-well microtiter plates. Another incubation period of 9 hours was carried out for bacterial biofilm formation. Thereafter, a series of two-fold dilutions of the substances were added to the wells containing the produced biofilm biomass.

The presumed antimicrobial activity of the tested essential oils was investigated by determining minimal inhibitory concentration values. To determine MIC values, the appropriate bacterial

suspension and the essential oil were added to the wells at the same time, which was followed by an incubation period of 24 hours at 37°C. After the incubation, MIC values were determined with the unaided eye.

Methods used to investigate antibiofilm effect

Crystal violet (CV) staining. To quantify total biofilm biomass formation, the supernatant was aspirated, and the wells were washed with physiological saline solution to remove slime and nonadherent cells. The formed biofilm biomass was stained with 0.1% crystal violet for 20 minutes at room temperature. Thereafter, the stain was discarded and the wells were washed with lukewarm tap water before adding ethanol: acetone 3:1 solution to solubilize the crystal violet. The absorbance of the biofilms was then measured spectrophotometrically at 595 nm with a microplate reader.

MTS-formazan assay. Another method for the examination of biofilm formation used in this study was completed with *CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay* (MTS) which was purchased from Promega (Madison, USA). Following the appropriate incubation, the supernatant was aspirated to eliminate planktonic cells, and wells were washed with sterile physiological saline solution to remove nonadherent cells. After washing, a dilution of MTS-formazan: MH broth 1:5 solution was added to the wells, which was followed by an incubation of 1,5 hours at 37°C. The volume of viable cells in the biofilm was determined colorimetrically by measuring the production of reduced MTS-formazan at 495 nm.

Results

As a result of the spectrophotometric measurements, absorbance values were obtained. The absorbance values were first corrected considering the negative controls (their absorbance was subtracted from the measured values), then compared to the positive control (which was considered as 100%). The reduction of the biofilm biomass was expressed as a percentage, and where the tested substance reduced the detection of the biofilm with at least 50%, EC_{50} value was determined.

NAC and cysteine derivatives

In our recent study, antibiofilm effect of NAC was not observed by crystal violet staining or MTS-formazan assay. N-monochloroacetyl-cysteine decreased the volume of biofilm biomass (from 16 mg/ml), and resulted in a reduction in the number of viable cell counts embedded in the biofilm (from 8 mg/ml) produced by *P. aeruginosa* strains. Excellent results were observed in the case of *S. aureus* strains as well, N-monochloroacetyl-cysteine significantly decreased the amount of viable cells from 8 mg/ml. N-dichloroacetyl-cysteine and N-benzoylcysteine both showed a slightly observable effect on *P. aeruginosa* biofilms at a concentration of 32 mg/ml, but did not affect *S. aureus* biofilms.

mg/ml	**32**	**16**	**8**	**4**	**2**	**+**
1.	84%	97%	104%	111%	104%	100%
2.	107%	146%	157%	152%	118%	100%
3.	82%	94%	105%	106%	103%	100%
4.	98%	110%	114%	112%	112%	100%

The effect of different concentrations of **NAC (mg/ml)** against biofilms produced by 4 *S. aureus* strains, examined by MTS-formazan assay. No EC_{50} could be determined.

mg/ml	32	16	8	4	2	+
1.	56%	31%	36%	135%	126%	100%
2.	56%	29%	29%	161%	143%	100%
3.	126%	58%	144%	199%	106%	100%
4.	48%	33%	34%	135%	112%	100%

The effect of different concentrations of **N-monochloroacetyl-cysteine** (mg/ml) against biofilms produced by 4 *S. aureus* strains, examined by MTS-formazan assay. Treated wells with a reduction in the biofilm of at least 50% were marked with a dark yellow background. The corresponding concentration is considered to be the EC_{50} value in our recent study.

mg/ml	32	16	8	4	2	+
1.	170%	90%	202%	165%	137%	100%
2.	170%	93%	217%	150%	123%	100%
3.	176%	121%	289%	150%	83%	100%
4.	111%	70%	191%	144%	110%	100%

The effect of different concentrations of **N-dichloroacetyl-cysteine** (mg/ml) against biofilms produced by 4 *S. aureus* strains, examined by MTS-formazan assay. No EC_{50} could be determined.

mg/ml	32	16	8	4	2	+
1.	215%	166%	150%	128%	104%	100%
2.	177%	152%	145%	145%	123%	100%
3.	219%	178%	153%	166%	124%	100%
4.	198%	181%	138%	129%	122%	100%

The effect of different concentrations of N-benzoylcysteine against biofilms produced by 4 *S. aureus* strains, examined by MTS-formazan assay. No EC_{50} could be determined

Essential oils

All the studied essential oils significantly reduced the produced biofilm biomass of the tested bacterial strains. Thyme oil had a strong effect on *S. aureus* biofilms and caused a significant decrease in the biofilm biomass from 0,15 mg/ml. Higher concentrations (from 2,5 mg/ml) were

required for the reduction of *P. aeruginosa* biofilms. Cinnamon oil reduced the volume of the formed *S. aureus* biofilms at a concentration of 0,45 mg/ml. *P. aeruginosa* biofilms were decreased from a concentration of 0,225 mg/ml. Formed biofilms of all the tested bacterial strains were impaired by geraniol at a concentration of 0,45 mg/ml.

Essential oils were also proved to be excellent antibacterial agents. The minimal inhibitory concentration of thyme oil was 0,15 mg/ml against *S. aureus* strains and 2,5 mg/ml against *P. aeruginosa* strains. Cinnamon oil inhibited the growth of all *S. aureus* and *P. aeruginosa* strains at 0,12 mg/ml. The minimal inhibitory concentration of geraniol against *S. aureus* strains was 0,225 mg/ml, while it was effective only at a concentration of 0,9 mg/ml against *P. aeruginosa* strains.

mg/ml	**3,6**	**1,8**	**0,9**	**0,45**	**0,225**	**0,12**	**0,06**	**0,03**	**0,015**	**0,008**	**+**
1.	7%	5%	4%	16%	131%	118%	102%	103%	84%	104%	100%
2.	4%	1%	2%	10%	158%	128%	128%	108%	126%	93%	100%
3.	4%	2%	1%	9%	101%	111%	106%	114%	113%	101%	100%
4.	2%	1%	1%	2%	57 %	71%	81%	84%	87%	103%	100%

The effect of different concentrations of geraniol against biofilms produced by 4 *S. aureus* strains, examined by MTS-formazan assay. Treated wells with a reduction in the biofilm of at least 50% were marked with a dark yellow background. The corresponding concentration is considered to be the EC_{50} value in our recent study.

mg/ml	**7,2**	**3,6**	**1,8**	**0,9**	**0,45**	**0,225**	**0,12**	**0,06**	**0,03**	**0,015**	**+**
1.	6%	5%	5%	15%	68%	91%	96%	117%	120%	115%	100%
2.	3%	5%	5%	13%	54%	40%	66%	99%	91%	101%	100%
3.	3%	3%	3%	10%	48%	59%	80%	81%	102%	81%	100%
4.	4%	2%	3%	9%	32%	38%	99%	85%	103%	111%	100%

The effect of different concentrations of cinnamon oil against biofilms produced by 4 *S. aureus* strains, examined by MTS-formazan assay. Treated wells with a reduction in the biofilm of at least 50% were marked with a dark yellow background. The corresponding concentration is considered to be the EC_{50} value in our recent study.

mg/ml	40	20	10	5	2,5	1,25	0,625	0,313	0,156	0,078	+
1.	9%	26%	8%	2%	1%	1%	0,5%	0,5%	7%	80%	100%
2.	6%	20%	5%	2%	1%	0,5%	0%	0%	2%	53%	100%
3.	12%	31%	9%	2%	1%	0,5%	0%	0,5%	56%	95%	100%
4.	13%	13%	6%	2%	1%	0,5%	0%	0,5%	20%	60%	100%

The effect of different concentrations of thyme oil against biofilms produced by 4 *S. aureus* strains, examined by MTS-formazan assay. Treated wells with a reduction in the biofilm of at least 50% were marked with a dark yellow background. The corresponding concentration is considered to be the EC_{50} value in our recent study.

Plant-derived non-essential oil extracts

Rosemary, mint, green tea extracts and baicalin were not effective against *P. aeruginosa* or *S. aureus* biofilms. Slight damage could be observed on *P. aeruginosa* biofilms treated by water chestnut extract at a concentration of 2 mg/ml.

mg/ml	2	1	0,5	0,25	0,125	+
1.	7%	92%	93%	92%	97%	100%
2.	76%	98%	220%	204%	268%	100%
3.	19%	90%	92%	91%	97%	100%
4.	11%	68%	44%	57%	101%	100%

The effect of different concentrations of water chestnut extract against biofilms produced by 4 *P. aeruginosa* strains, examined by crystal violet staining. Treated wells with a reduction in the biofilm of at least 50% were marked with purple background. The corresponding concentration is considered to be the EC_{50} value in our recent study.

Tryptophan

Tryptophan had no observable effect on *P. aeruginosa* or *S. aureus* biofilms in the tested concentrations.

mg/ml	0,816	0,408	0,204	0,102	0,051	+
1.	92%	102%	115%	105%	100%	100%
2.	251%	219%	217%	170%	120%	100%
3.	240%	83%	100%	110%	112%	100%
4.	590%	100%	404%	272%	145/	100%

The effect of different concentrations of tryptophan against biofilms produced by 4 *P. aeruginosa* strains, examined by crystal violet staining.

EDTA

Excellent results were carried out by treating formed biofilms of *P. aeruginosa* and *S. aureus* strains with EDTA. It was effective in reducing the produced biofilm biomass even in extremely low concentrations (from 0,03 mg/ml).

mg/ml	26,1	13,05	6,525	3,26	1,63	0,815	0,407	0,102	0,051	0,0254	+
1.	19%	17%	20%	35%	67%	78%	107%	101%	92%	93%	100%
2.	43%	42%	46%	78%	76%	71%	113%	88%	88%	89%	100%
3.	31%	38%	47%	94%	112%	117%	77%	66%	80%	67%	100%
4.	13%	25%	22%	31%	44%	38%	65%	102%	110%	97%	100%

The effect of different concentrations of EDTA against biofilms produced by 4 *P. aeruginosa* strains, examined by MTS-formazan assay. Treated wells with a reduction in the biofilm of at least 50% were marked with a dark yellow background. The corresponding concentration is considered to be the EC_{50} value in our recent study.

4. INVESTIGATION OF SYNERGY BETWEEN COMBINATIONS OF ANTIMICROBIAL AND ANTIBIOFILM SUBSTANCES

A special microtiter plate layout was carried out to test the efficacy of combinations, where the effects of the substances against biofilms could be studied alone and in combination at different concentrations. Investigation of the synergism between the substances was tested on 1 moderate biofilm-producer *P. aeruginosa* strain, isolated from canine otitis externa. Reduction in the amount of viable cells embedded in the biofilm was determined by MTS-formazan assay, while a decrease in the volume of biofilm biomass was detected by crystal violet staining. The antimicrobial effect of the combinations was studied by determination of MIC. In order to describe interactions between the investigated substances, the checkerboard microdilution method was utilized for determining the lowest fractional inhibitory concentration (FIC) index. Similar to the single antibiotic susceptibility tests, bacterial strains were distributed in the microtiter wells to achieve a 10^5 CFU/mL bacterial density. The combined effects of the antibiotics were evaluated as synergy, addition, indifference, and antagonism.

A combination of geraniol and EDTA was proved to be most effective against the tested *P. aeruginosa* strain with FIC values showing full synergy. Marbofloxacin and EDTA together also showed excellent results. With the addition of EDTA, the sufficient dose of marbofloxacin against the tested bacterial strain was considerably lower. The combination of marbofloxacin and geraniol represented less synergistic effects than the two other combinations.

The combination of EDTA and NAC was also tested against 12 *P. aeruginosa* strains. The synergism between the two substances was observable, as far better results were obtained against each strains when treated with the combination than using the two substances separately. Selected results are shown in Figure 7 on a *P. aeruginosa* isolate.

Appendix

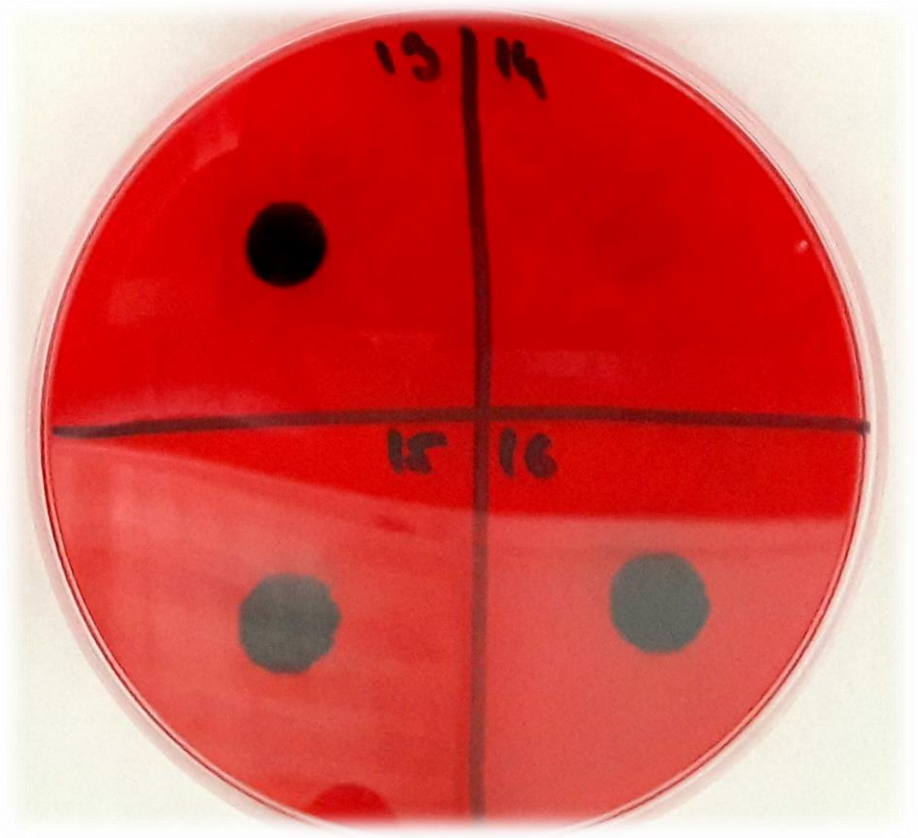
1. The intensity of biofilm formation of 73 *P. aeruginosa* strains isolated from canine otitis externa. The produced biofilm biomass was stained with crystal violet, then the absorbance was measured spectrophotometrically.

Number of strain	Absorbance (average)	Spread	Group
1.	0,171333	0,036341	weak
2.	0,4475	0,125266	weak
3.	1,012	0,246142	moderate
4.	0,491333	0,052206	weak
5.	0,960667	0,15015	moderate
6.	0,2075	0,056181	weak
7. (ATCC+)	1,775667	0,594144	strong
8. (ATCC-)	0,100167	0,019219	weak
9.	1,267667	0,281648	moderate
10.	1,318167	0,382282	moderate
11.	0,736167	0,229797	weak
12.	0,104833	0,028081	weak
13.	0,418833	0,067312	weak
14.	1,2285	0,668946	moderate
15.	0,797833	0,225809	weak
16.	0,123333	0,056216	weak
17.	1,94	0,501138	strong
18.	0,219667	0,320468	weak
19.	1,522667	0,49186	moderate
20.	0,08	0,021744	non-biofilm producer
21.	0,685167	0,203038	weak
22.	0,085667	0,014774	non-biofilm producer
23.	0,073167	0,00806	non-biofilm producer
24.	0,073167	0,012952	non-biofilm producer
25.	1,417333	0,18122	moderate
26.	0,4495	0,280564	weak
27.	0,414333	0,109653	weak
28.	1,706667	0,654021	moderate
29.	0,911333	0,254274	moderate

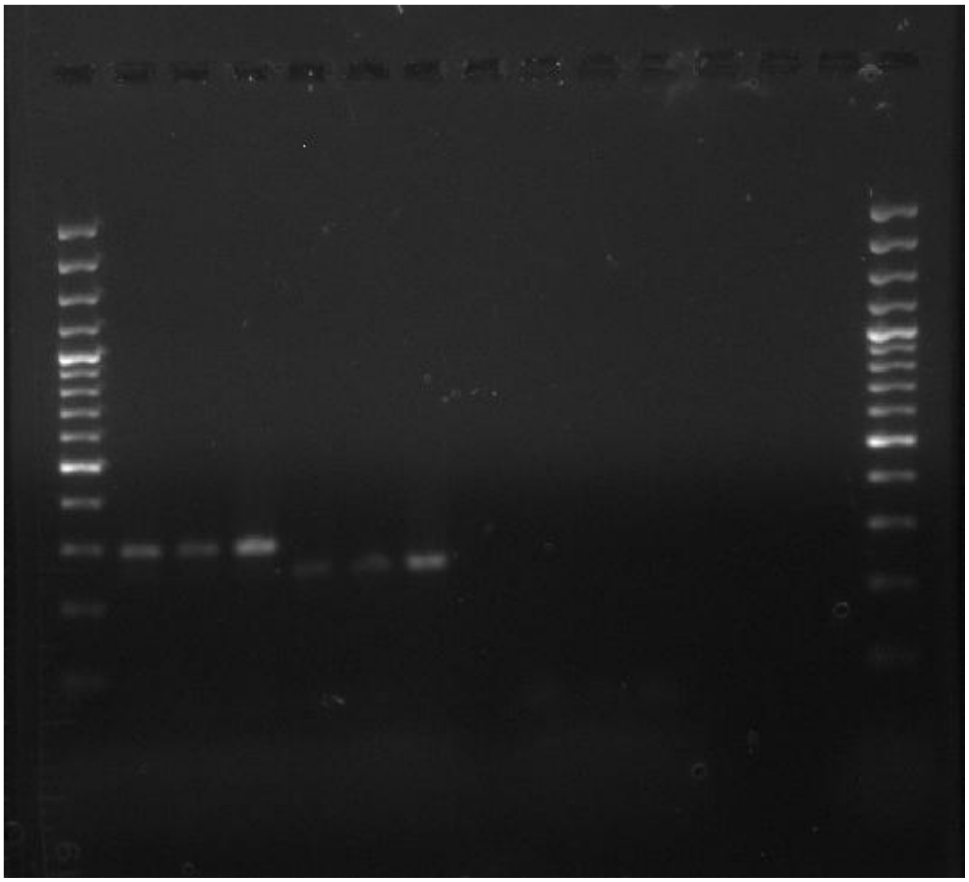
30.	1,611667	0,485903	moderate
31.	0,1265	0,041918	weak
32.	3,440333	0,432423	strong
33.	1,848333	0,377457	strong
34.	0,984667	0,29933	moderate
35.	1,136333	0,248046	moderate
36.	0,241167	0,22409	weak
37.	1,096167	0,427443	moderate
38.	0,098333	0,012372	non-biofilm producer
39.	2,286333	0,91351	strong
40.	2,741167	0,722065	strong
41.	0,653333	0,093637	weak
42.	0,1275	0,029898	weak
43.	0,264167	0,093579	weak
44.	2,239167	0,570879	strong
45.	2,381333	0,133814	strong
46.	1,307667	1,233136	moderate
47.	1,513167	1,418567	moderate
48.	1,070333	0,470535	moderate
49.	1,671	0,292597	moderate
50.	0,575667	0,173061	weak
51.	4,158833	0,905656	strong
52.	0,080333	0,025097	non-biofilm producer
53.	3,348333	0,307711	strong
54.	0,334333	0,052671	weak
55.	0,951333	0,089701	moderate
56.	1,3305	0,312253	moderate
57.	1,328833	0,270911	moderate
58.	1,641167	0,287439	moderate
59.	1,2385	0,277426	moderate
60.	0,694333	0,156015	weak
62.	2,6935	1,193535	strong
63.	2,094667	0,711225	strong
64.	0,729	0,093559	weak
65.	0,384667	0,080007	weak

66.	3,202	0,739575	strong
67.	0,156833	0,069497	weak
68.	0,456167	0,093088	weak
69.	1,416333	0,600765	moderate
70.	2,356	2,325998	strong
71.	1,122	0,239453	moderate
72.	0,596833	0,150039	weak
73.	0,379	0,206061	weak

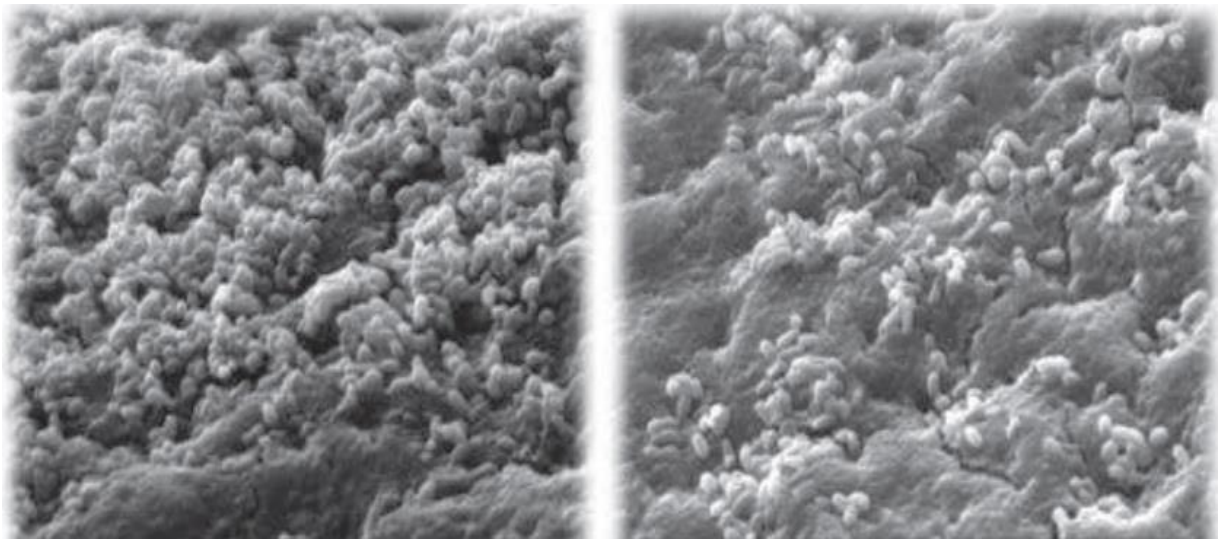
2. Congo red agar method was used to determine biofilm formation of *S. pseudintermedius* isolates. Strains forming black and dark gray colonies were considered as biofilm producers, while non-biofilm producers were red in colour.



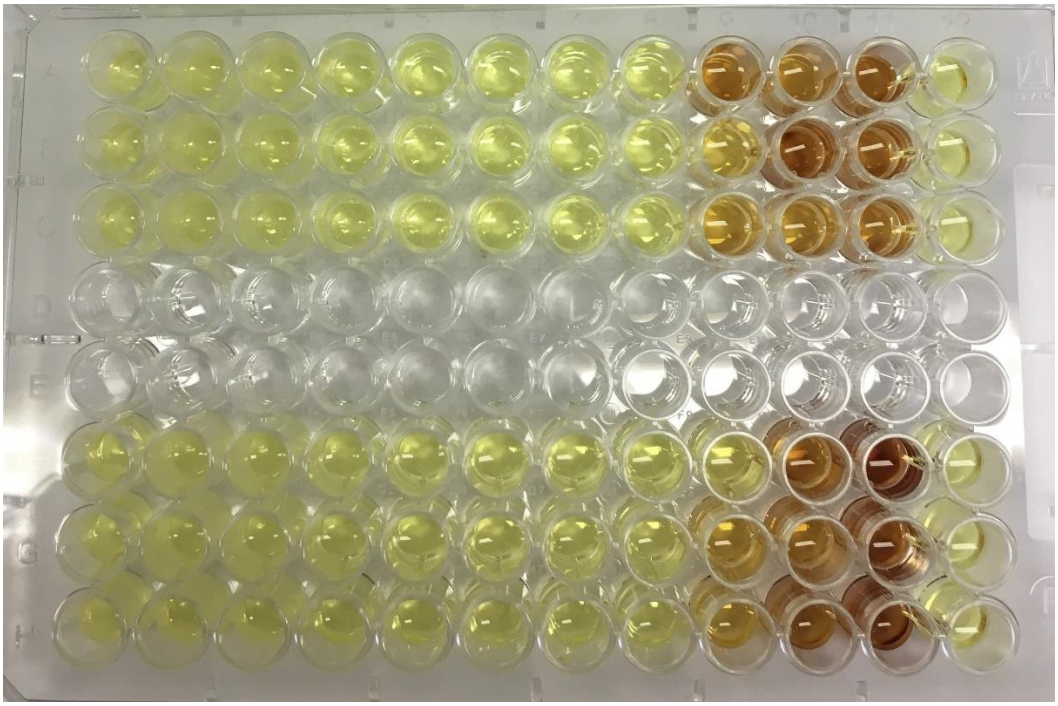
3. Detection of *pelA, pelC, pelE* (left to the right) genes by PCR method in *P. aeruginosa* strains isolated from canine otitis externa.



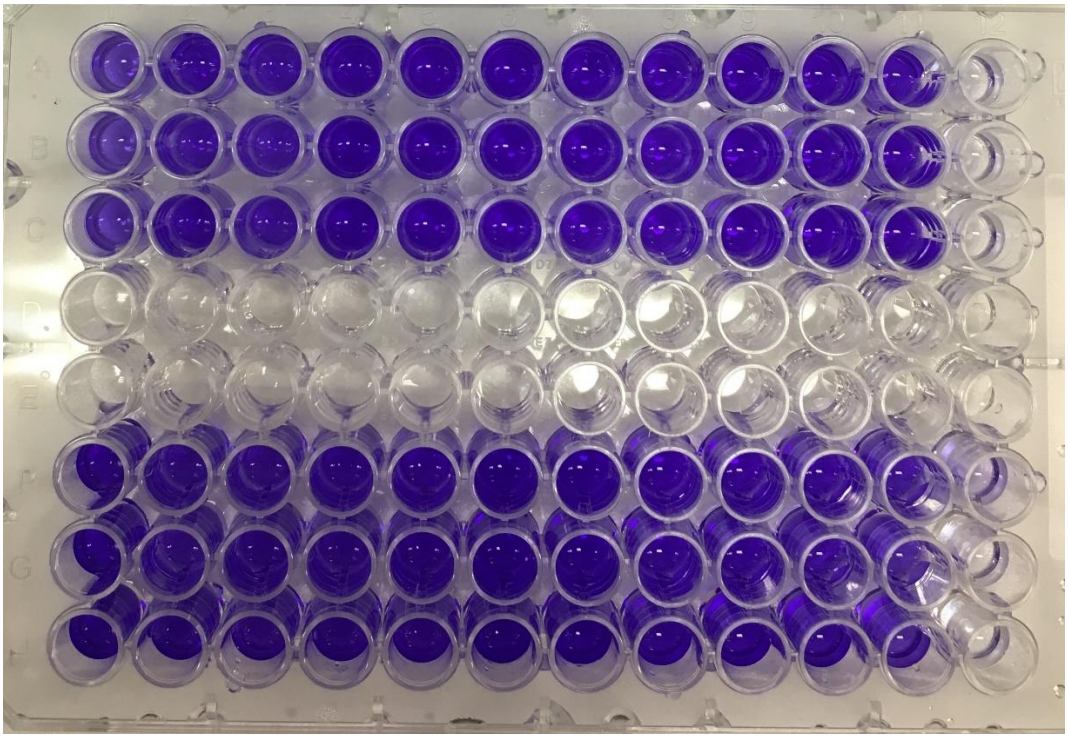
4. Scanning electron microscopy permits the imaging of bacterial cells embedded in the polysaccharide matrix. These photos show the produced biofilms of two different *P. aeruginosa* strains.



5. Investigation of the effect of thyme oil on biofilms of two different *S. aureus* strains, examined by MTS-formazan assay.



6. Investigation of the effect of NAC on biofilms of two different *S. aureus* strains, examined by crystal violet staining.



7. Investigation of synergistic effects between combinations of antimicrobial and antibiofilm substances against 1 moderate biofilm-producer *P. aeruginosa* strain. Tables show the reduction in the biofilm in percentage after performing MTS-formazan assay.

	Marbofloxacin (µg/ml)								
Geraniol (mg/ml)		**4**	**2**	**1**	**0,5**	**0,25**	**0,126**	**0,06**	**0,03**	**+**
	3,3	18%	18%	18%	17%	15%	16%	17%	15%	100%
	1,65	14%	13%	13%	13%	25%	25%	53%	57%	100%
	0,83	14%	13%	14%	13%	23%	34%	39%	54%	100%
	0,42	15%	15%	16%	18%	34%	30%	67%	69%	100%
	0,21	18%	18%	26%	29%	35%	35%	62%	70%	100%
	0,11	20%	20%	30%	29%	67%	86%	96%	96%	100%
	0,05	20%	22%	40%	56%	88%	133%	133%	130%	100%
	0,025	18%	36%	46%	73%	132%	147%	132%	131%	100%

	Marbofloxacin (µg/ml)								
EDTA (mg/ml)		**4**	**2**	**1**	**0,5**	**0,25**	**0,126**	**0,06**	**0,03**	**+**
	8	25%	21%	20%	25%	21%	29%	21%	30%	100%
	4	30%	31%	22%	27%	23%	26%	25%	31%	100%
	2	36%	31%	26%	29%	32%	67%	63%	72%	100%
	1	35%	32%	31%	36%	42%	57%	68%	79%	100%
	0,5	28%	37%	38%	41%	46%	54%	67%	103%	100%
	0,25	28%	33%	38%	49%	62%	66%	92%	112%	100%
	0,125	27%	33%	42%	49%	74%	81%	90%	95%	100%
	0,0625	20%	25%	37%	91%	101%	105%	104%	113%	100%

	Geraniol (mg/ml)								
EDTA (mg/ml)		**3,3**	**1,65**	**0,83**	**0,42**	**0,21**	**0,11**	**0,05**	**0,025**	**+**
	8	9%	9%	9%	9%	8%	9%	13%	21%	100%
	4	8%	8%	8%	8%	8%	9%	15%	24%	100%
	2	8%	8%	8%	8%	7%	10%	18%	79%	100%
	1	8%	8%	8%	7%	7%	10%	34%	89%	100%
	0,5	8%	8%	8%	7%	7%	19%	60%	73%	100%
	0,25	8%	8%	7%	7%	7%	44%	102%	103%	100%
	0,125	8%	7%	7%	8%	7%	64%	94%	110%	100%
	0,0625	10%	9%	48%	76%	84%	89%	107%	97%	100%