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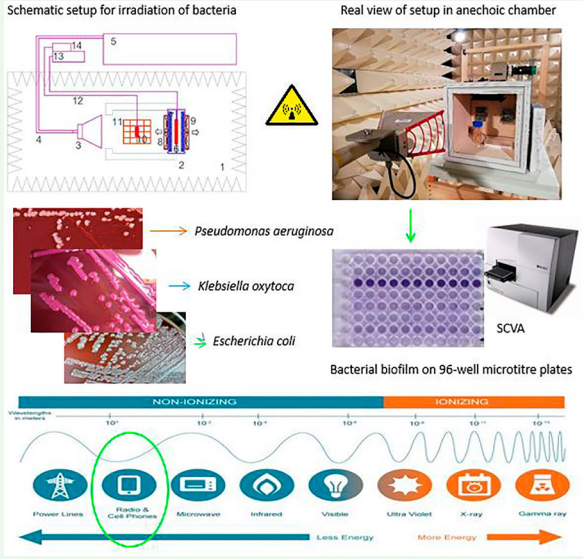
The effect of exposure to non-ionising radiofrequency field on *Escherichia coli*, *Klebsiella oxytoca* and *Pseudomonas aeruginosa* biofilms

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ABSTRACT

An environment with a higher accumulation of electromagnetic non-ionising radiofrequency (RF) emissions generated by various telecommunication, data transport and navigation devices (mobile phones, Wi-Fi, radar, etc.) may have a major impact on biological systems. This study aimed to evaluate the incidence of an electromagnetic field (EMF) on the development of bacterial biofilm. Quantification of biofilm production was done by using microtiter plate assay. Bacterial isolates of *Escherichia coli*, *Klebsiella oxytoca* and *Pseudomonas aeruginosa* were exposed with EMF of frequencies 1–5 and 2.4 GHz with an exposure time 3 or 24 h, respectively. Exposure of bacteria to EMF produced a statistically significant increase in biofilm production mainly at 1, 2 and 4 GHz, and in contrast, a significant inhibition of biofilm development occurred at frequencies 3 and 5 GHz, both with exception of *K. oxytoca* and *P. aeruginosa*. Wi-Fi operating at 2.4 GHz caused biofilm reduction. The results indicate that EMF exposure act on bacteria in both ways, depending on the frequency: as stressful by enhancing bacterial biofilm formation (under environmental stress, bacteria produce a polysaccharide matrix and aggregate to form biofilms to increase virulence and resistance), although some frequencies leading to biofilm damage could be caused by changes to the physicochemical properties of bacteria.



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Introduction

Viruses are these days an intensively observed topic, although multi-resistant biofilm-associated bacteria and related problems with treatment are not losing the interest of scientists and engineers. The past decade has brought and the coming decade will continue to bring new technologies, such as the Internet of Things (IoT), new generations of mobile networks (5G), higher performance in wireless information transmission and others. These technologies, based on the radiofrequency (RF) spectrum of an electromagnetic field (EMF) create an unnatural environment for

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people, animals or other biological systems and are known to interact differently with living organisms through either thermal or non-thermal effects, depending on the individual parameters.

Places with a cumulative occurrence of mobile phones, laptops or other wireless devices (at airports, onboard aircraft, at shopping malls, offices) can be considered in particular as localities with a higher EMF density. As long as a concentration of people is present, moreover coming from various countries, there is a high chance of not only virus transmission but also different spectra of bacteria being present [1]. With a longer time spent in such an environment it is interesting to observe the behaviour of bacteria under these stressful conditions, which could affect their performance, biofilm formation or their resistance to antibiotics.

Several scientific studies devoted to biological materials exposed to EMF refer to the significant influence of EMF, with a demonstrable impact on the receptors of cell membranes and proteins. The energy produced by EMF is not considered strong enough to induce direct chemical changes in the cell constitutes, thus, the biological side effects of EMF are most likely imposed indirectly [2–4]. Many biological systems consist of charged gradients across membranes that fluctuate at certain frequencies and make them sensitive to EMF in a thermal or non-thermal manner. Low-frequency EMF (<400 KHz) disrupts ion diffusion while EMFs at high frequency (1 MHz–10 GHz) affect the molecular motion of water and intramolecular groups of biological macromolecules causing resonant vibrational or rotational motion. Thus, EMF affects the folding and unfolding status and dynamics of some globular proteins [5]. Exposure to EMFs can cause changes in the conformation of biologically active macromolecules including ion channels through water molecules. These channels may be found, e.g. on the outer membranes of *Escherichia coli* and they are substantially voltage sensitive. As such biological systems consist of charged and uncharged polyelectrolytes as well as ions with dynamic parameters, such as permittivity, potential, dipole moment and others, they are located in a polar aqueous medium, and this whole is sensitive to EMF [6].

Some scientific works concern the non-thermal effects of EMF on different bacterial strains in regard to various properties – growth, biofilm, antibiotic resistance, etc. [7–9]. Non-thermal EMF induced different responses on bacteria depending on the frequency intensity, exposure time and organism model [10]. Regarding bacterial biofilm, as described in Bucko et al. [7], exposure of *Staphylococcus aureus* to EMF with a power of 52.7 mW and frequencies of 1, 2 and 3 GHz revealed mostly the inhibition of biofilm development activity; however, a significant accumulation of biofilm occurred at frequencies 4 and 5 GHz. Said-Salman et al. [11] described a stimulation effect of 2.4 GHz Wi-Fi exposure on *E. coli* O157H7, *S. aureus* and *Staphylococcus epidermidis* biofilms; moreover, the mentioned authors, by using high-throughput RNA-sequencing, revealed 101 genes involved in metabolic pathways, transposition, response to stimuli, motility, chemotaxis and cell adhesion differentially expressed in *E. coli* K-12 DH5α exposed to 2.4 GHz [12].

The various effects of different types of EMF obtained so far, among them increasing bacterial biofilm formation, antibiotic resistance, motility, etc. (bacterial traits associated with pathogenicity), may be considered a worldwide threat to public health [9,11–14]. Moreover, regarding the well-documented 'window' effect, it is necessary to perform additional work to gather more information about changes in different bacterial types exposed to a variety of EMF.

Based on this, the aim of our study was to analyse the non-thermal influences of EMF (at frequencies 1, 2, 2.4, 3, 4 and 5 GHz) emitted by mobile phones and Wi-Fi routers, which are among those that frequently occur and create an unnatural environment for biological systems, including microorganisms, on the ability of Gram-negative bacteria (isolated from different sources, namely *E. coli, Klebsiella oxytoca* and *Pseudomonas aeruginosa*) to form biofilm.

Material and methods

Bacterial strains

The clinical isolate *P. aeruginosa* no. 44 originating from bovine lungs was obtained from the Veterinary and Food Institute (Dolný Kubín, Slovakia). *K. oxytoca* no. 44 was kindly provided by Dr L. Bírošová from the Slovak University of Technology, Department of Nutrition and Food Quality Assessment in Bratislava and was acquired from a smoothie.

E. coli no. 2 and 14 were isolated from poultry, no. 3 from goshawk, no. J2 from heifer and no. 24 from piglet (faeces). The majority of isolates showed multiresistance, aside from *E. coli* no. 24 and *K. oxytoca* no. 44 (susceptible strains). All strains were cultured at 37° C overnight on Brain Heart Infusion (BHI) agar (Oxoid, Basingstoke, United Kingdom).

Electromagnetic RF radiation

For our experiment, an RF anechoic chamber by Frankonia with a working frequency range from 10 kHz to

18 GHz was used. This was the way of filtering out fields with different frequencies. Some works [12,15] used a Wi-Fi router itself as a source of EMF for experiments, although a sample may be RF irradiated by EMF with different frequencies or intensities coming from other sources of radiation (other Wi-Fi routers, phones).

A part of the anechoic chamber was a temperature box based on the principle of DC Peltier thermocouples, and the heat was conducted to the sample space with the help of aluminium radiators and ventilators. The temperature was regulated by a thermostat at 37°C. A funnel antenna connected to the signal generator by a shielded coaxial cable was used as a source of non-ionising RF spectrum EMF. A Rohde & Schwarz SMA 100A signal generator with a frequency range of 9 kHz to 6 GHz with a gain from −145dBm to +18dBm was used.

The samples were exposed to EMF with frequencies of 1, 2, 3, 4 and 5 and 2.4 GHz and gain 17.2dBm for a period of 3 or 24 h. The frequencies were selected from the frequency range based on the devices used in telecommunication networks (mobile phone) and for data transfer (Wi-Fi).

Biofilm formation

Biofilm production was performed using spectrometric crystal violet assay (SCVA) by a method previously published in O'Toole et al. [16] with slight modifications according to Čuvalova et al. [17]. In brief, bacterial isolates (*E. coli*, *K. oxytoca* and *P. aeruginosa*) were grown on BHI agar (Oxoid, Basingstoke, United Kingdom), and the colonies were transferred to BHI broth (Oxoid, Basingstoke, United Kingdom) to reach a density equivalent to $1.5–3 \times 10^8$ CFU/mL. A volume of 200 µL of these bacterial suspensions was inoculated in the wells of 96-well Nunc polystyrene microtiter plates (Thermo Fisher Scientific, Roskilde, Denmark) and followed by exposure to individual non-ionising RF spectrum of an electromagnetic field of frequencies (1–5 GHz) and/or 2.4 GHz for 3 or 24 h at 37°C. In the case of RF irradiation for 3 h, biofilms incubation was continued until reaching the required time for biofilm development – overnight growth. Our experiment was interested in evaluating the EMF effects on biofilm formation (the initial biofilm stage). After incubation, sessile bacteria were washed three times with a phosphate-buffered saline solution (PBS, Thermo Fisher Scientific), fixed with methanol for 15 min, dried and stained with a 0.1% crystal violet (Mikrochem, Pezinok, Slovak Republic) solution for 15 min. The adhering dye was dissolved with 33% acetic acid. Eight replicates were used for each test exposed/control. The results were obtained by measurement of absorbance at $\lambda = 550$ nm in a Synergy HT Multi-Mode Microplate Reader (BioTek, Winooski, Vermont, United States of America).

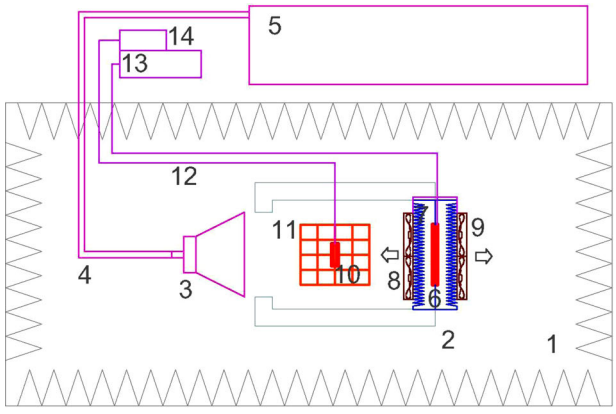
Statistical analysis

Biofilm experiments were statistically analysed using the software package Statistica AXAZ (StatSoftCR, Czech Republic). Bacterial biofilm formation was represented as the average of absorbance values measured at $\lambda =$ 550 nm (A_{550}) ± standard deviation (SD) per well of the 96-well Nunc polystyrene microtiter plates. One-way ANOVA and Tukey's *post-hoc* test were used to detect significant differences between control and RF-exposed bacterial biofilms, and statistical significance was accepted at $p < 0.05$ level.

Results and discussion

In order to explore the influence of different frequencies (1, 2, 2.4, 3, 4 and 5 GHz) of EMF on bacterial biofilm formation, the SCVA based on the values of optical density of the crystal violet stain bonded to the biofilm were performed. To obtain the exact results determining the explicitly non-thermal effect and the required frequency free of other devices, experiments were carried out in an RF anechoic chamber by Frankonia, with working frequency range from 10 kHz to 18 GHz as can be seen in Figure 1(b). The thermal effects of exposure to EMF frequencies were eliminated through the use of a regulator – a Peltier module between two aluminium radiators with ventilators for conducting heat/cool and an external heater capable of maintaining microplate temperature with the monitored bacteria at 37°C (± 1°C), relevant for biofilm cultivation (Figure 1(a)).

Data obtained on the development of *E. coli*, *K. oxytoca* and *P. aeruginosa* biofilm showed diverse feedback; unaffected, increased, or suppressed (Figures 2 (a–f); Table 1). Electromagnetic field of frequencies 1 and 2 GHz predominantly caused a significant enhancement of biofilm development (Figures 2(a,b); Table 1). A frequency of 4 GHz stimulated biofilm production by a significant amount in three *E. coli*: no. 3 from goshawk, no. 24 from piglet and no. J2 from heifer (in the aforementioned last isolate J2 from the value $A_{550} = 0.128 \pm 0.049$ in the control to $A_{550} = 0.295 \pm 0.110$ in the RF irradiated group, p-value < 0.01–57% expansion in biofilm); however, 4 GHz also caused significant inhibition of biofilm in *E. coli* no. 14 from poultry (from value of $A_{550} = 0.353 \pm 0.136$ in the control to $A_{550} = 0.187 \pm 0.090$ in the exposed bacteria, p-value < 0.05–47% decrease in biofilm) (Figure 2(d); Table 1).



a



b

Figure 1. Setup for RF irradiation of sample. (a) Schematic view of the setup used for RF irradiation of bacteria, where 1 – anechoic chamber, 2 – assembled temperature chamber, 3 – horn antenna connected by 4 – shield coax cable to 5 – signal generator, as source of heat was used 6 - Peltier module between 7 – two aluminium coolers with 8/9 – ventilators for conducting heat/cool, 10 – thermal sensor and 11 - sample holder was situated in middle of chamber. The temperature apparatus was connected by 12 – wires to 13 – DC source controlled by 14 – thermostat; (b) Real view of setup in RF anechoic chamber.

In contrast, electromagnetic field of frequencies 3 and 5 GHz showed mostly the inhibition of biofilm development, except in *K. oxytoca* no. 44 after exposure to 5 GHz with statistically significant biofilm stimulation occurring (from $A_{550} = 0.553 \pm 0.22$ to $A_{550} = 1.4 \pm 0.4$, p-value < 0.001–60% expansion in biofilm) (Figures 2(c,e); Table 1).

Contradictory results occurred after bacterial exposure to EMF of frequencies 2 and 3 GHz (stimulation versus inhibition); bacterial biofilms were exposed by the most frequently used wireless fidelity for data transfer operating at 2.4 GHz. The result achieved showed biofilm reduction in all the tested bacteria, with statistical significance in two *E. coli,* no. 14 and J2, and *K. oxytoca* no. 44 (Figure 2(f), Table 1). Our observation is in line with the previous reports of Mortazavi [18] who showed that radiation-induced stimulator/inhibitory effects can be observed only within a narrow window of radiation dose – the well-documented 'window' effect (different field parameters may result in different outcomes) [19].

The present study examined the effects of exposing *E. coli, K. oxytoca* and *P. aeruginosa* to EMF at frequencies of 1–5 GHz for three hours and the Wi-Fi of frequency 2.4 GHz continuously for 24 h on their ability to form biofilms. Despite the existence of various stages of biofilm growth mode (biofilm formation, attachment and pre-formed biofilm), our experiments focused on the effect of EMF in preventing initial biofilm formation. The important step in all biofilm development is the initial bacterial cell attachment to a surface. This step is critical for the formation of a bacterial biofilm, as all other cells within the biofilm structure rely on the interaction between surface and bacterial cell for their survival [20]. Given time, attached cells aggregate to form micro-colonies while excreting extracellular polymeric substances; the bottom-layer of the biofilm is thus considered established and continues to mature into a multi-layered cluster (and can be upwards of multiple micrometres in thickness). The embedded biofilm cells do not divide; instead, their excess energy is used to maintain the extracellular matrix, contributing to higher biofilm resilience [21]. As the so-called early stage of biofilm is a key step and the most decisive for the preservation of biofilm, its manipulation is crucial. Since the continuing stage of the biofilm is more protected from various chemical or physical influences caused by the presence of on extracellular matrix, we believe that in order to maintain the reduction of the biofilm after 24 h (the time of monitoring for 2.4 GHz), its influence in the early stages is responsible. Although we cannot guarantee that the results after 3 h will also copy our results after 24 h, we strongly believe that despite all, maintaining the inhibition of the mature more resistant biofilm after prolonged non-ionising RF of EMF is a positive result, ant it at least confirms that longer exposure of Wi-Fi EMF of frequency 2.4 GHz causes diminution in the biofilm and thus does not contribute to increasing the pathogenicity of the monitored bacteria.

Whereas the mentioned EMF frequencies commonly occur in an increased level at our environment and could adversely affect the behaviour of bacteria, we were interested in whether or not EMF supports the formation of biofilm, and thus whether or not it contributes to increasing bacterial pathogenicity. Indeed, biofilm infections are a significant problem in chronic and long-term infections, including those colonising medical devices and implants.

Previously published research articles have monitored the effects of a rotating magnetic field on *S.*

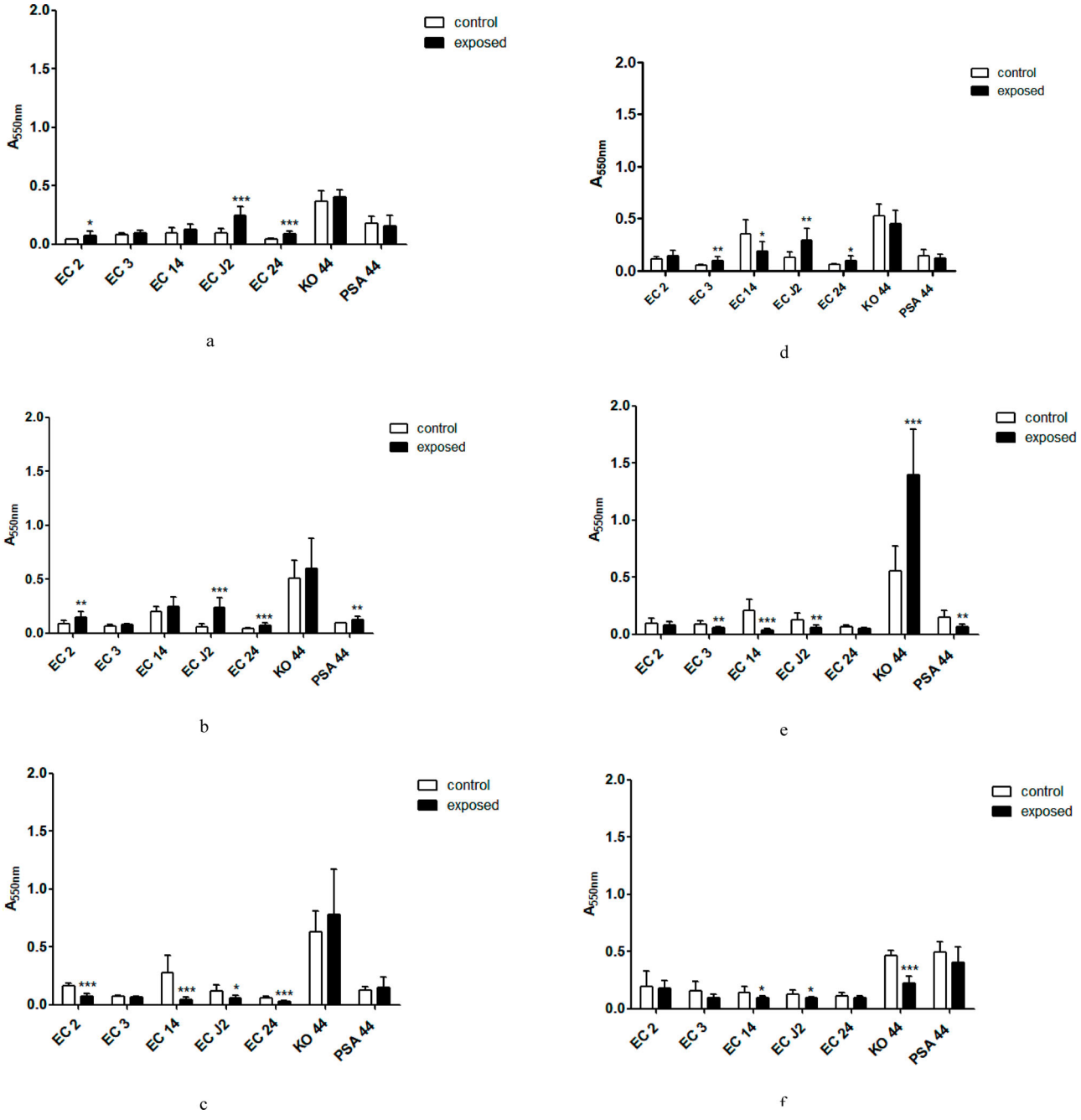


Figure 2. EMF of frequencies (1, 2, 2.4, 3, 4 and 5 GHz) effect on bacterial biofilm modulation: (a) 1 GHz; (b) 2 GHz; (c) 3 GHz; (d) 4 GHz; (e) 5 GHz; (f) 2.4 GHz. Legend: average values from eight replicate of absorbance; A_{550nm} – absorbance at $\lambda = 550$ nm of control and/or exposed groups; EC – *Escherichia coli*; KO – *Klebsiella oxytoca*; PSA – *Pseudomonas aeruginosa;* One-way ANOVA with Tukey's *post-hoc* test was used for statistical analysis between control and exposed groups, ***p-value < 0.001, **p-value < 0.01; *p-value < 0.05.

aureus and *E. coli* biofilm [22] or long-time (24 and 48 h) 2.4 GHz exposure on *E. coli* 0157H7, *S. aureus* and *Staphylococcus epidermis* biofilm formation [11], and the obtained results showed either no significant difference in the amount of biofilm produced or a statistically significant expansion in the biofilm. In contrast, in our experiments, application of 2.4 GHz overnight exposure to *E. coli, K. oxytoca* and *P. aeruginosa* caused a diminution in biofilm and confirmed that for the same field parameters cell type-specific responses and other parameters may be involved [23].

A previous study by Oncul et al. [14] revealed changes in the physicochemical properties of both Gram-positive and Gram-negative bacteria under the effect of extremely low-frequency electromagnetic fields (ELF-EMF). The ELF-EMF seems to affect the surface charge in a contrary manner in Gram-positive versus Gram-negative bacteria; however, it decreased the hydrophobicity of

Table 1. EMF of frequencies (1, 2, 2.4, 3, 4 and 5 GHz) effect on bacterial biofilm.

	EMF of frequencies (in GHz)					
Bacterial strains	1	2	2.4	3	4	5
Escherichia coli 2	*↑	**↑	n.s. ↓	***↓	n.s.↑	n.s.↓
Escherichia coli 3	n.s.↑	n.s.↑	n.s. ↓	n.s↓	**↑	**↓
Escherichia coli 14	n.s.↑	n.s.↑	*↓	***↓	*↓	***↓
Escherichia coli J2	***↑	***↑	*↓	*↓	**↑	**↓
Escherichia coli 24	***↑	***↑	n.s. ↓	***↓	*↑	n.s.↓
Klebsiella oxytoca 44	n.s. ↑	n.s.↑	***↓	n.s.↑	n.s.↓	***↑
Pseudomonas aeruginosa 44	n.s.↓	**↑	n.s. ↓	n.s.↑	n.s.↓	**↓

Note: Statistically significant bacterial biofilm stimulation (↑) /statistically significant bacterial biofilm inhibition (↓); One-way ANOVA with Tukey's post hoc test was used for statistical analysis between control and exposed groups, *** p value < 0.001, ** p value < 0.01; * p value < 0.05.

both monitored bacteria *S. aureus* and *E. coli.* Since cell surface hydrophobicity and surface charge are important for bacterial adhesion and defence mechanisms in host environments, it can be concluded that exposure to EMF could affect the behaviour of bacteria. However, this change will be closely related to the 'window' effect, time exposure and bacterial cells' type-specific responses.

Conclusions

In conclusion, the results of our study revealed that EMF can stimulate/inhibit the ability of *E. coli, K. oxytoca* and *P. aeruginosa* to form biofilms, depending on the frequency and/or specific type of bacteria used. Based on the acquired results of the biofilm modulation (increasing/decreasing) EMF can also be expected to influence the pathogenicity of microorganisms, since biofilm-associated bacteria are considered more resistant and difficult to treat; however, some results demonstrated EMF as a potential tool for biofilm removal, for example, on medical devices and biomaterials. Further studies are in progress (and need to be performed) to extend the current state of knowledge of the influence of EMF on microorganisms and to gather more information on other bacterial biological changeovers that may occur due to exposure to EMF, including antibiotic susceptibility (resistance) as a next substantial factor of bacterial pathogenicity. Undervaluing the problem of exposure to electromagnetic non-ionising RF radiation generated by various telecommunication and data transport as common part of the environment could lead to a further rise in infectious diseases or their complications.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability statement

The authors confirm that the data supporting the findings of this study are available within the article.

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