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Impact of sodium bicarbonate, boric, medium mineralization highly carbonated water and ciprofloxacin combination on the some upper respiratory tract microbiota biofilm architecture

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Abstract

Introduction and Objective. The use of irrigation therapy as an adjunctive treatment for upper respiratory tract infections is attracting interest. This is particularly notable when the effectiveness of conventional antibiotic therapy is reduced. The aim of this study is investigation of effects of sodium bicarbonate, boric, medium mineralization highly carbonated water, alone and in combination with Ciprofloxacin, on the architecture and intensity of biofilm formation by representative upper respiratory tract microbiota.

Materials and Method. Multispecies biofilms consisting of Lactobacillus sporogenes co-cultured with Escherichia coli ATCC 25922 or Staphylococcus aureus ATCC 25923 were used as experimental models. Biofilm formation was quantified using crystal violet staining and spectrophotometric analysis, while structural characteristics were assessed by light microscopy and morphometric image analysis.

Results. Ciprofloxacin at minimum inhibitory concentrations significantly increased biofilm biomass and coverage in both microbial consortia, indicating antibiotic-induced biofilm enhancement. Treatment with mineral water alone reduced total biofilm area and coverage, but promoted fragmentation into a greater number of discrete microcolonies.

Conclusions. Mineral water modulates biofilm cohesion and spatial organization rather than solely reducing microbial viability. The study demonstrates the potential of mineral water as an adjunctive agent to enhance antibiofilm efficacy in the management of antibiotics in upper respiratory tract infection

Key words

biofilm, mineral water, Ciprofloxacin, elimination-irrigation therapy, upper respiratory tract microbiota

INTRODUCTION AND OBJECTIVE

Microbial biofilms, structured communities of their cells encased in an extracellular matrix, are constant on the any human mucosa and skin surface. The composition of the biofilm microbiota varies between anatomical niches. The upper respiratory tract, comprising the nasal cavity, nasopharynx, oropharynx, and larynx, is colonized by a diverse and dynamic community of such microorganisms that for human heath are collectively referred to as the normal microbiota. This microbiota includes commensal bacteria, viruses, and fungi that contribute to both local and systemic immune homeostasis. The bacterial members of this community are particularly well studied, with predominant genera including *Streptococcus*, *Staphylococcus*, *Corynebacterium*, *Moraxella*, *Neisseria*, *Haemophilus* and *Dolosigranulum* [1]. The nasal cavity is dominated by *Corynebacterium spp.* and coagulase-negative *Staphylococcus spp.*, whereas the nasopharynx harbours more dynamic communities influenced by age, environment, and health status. In infants and young children, the colonization pattern is strongly shaped by the mode of delivery, breastfeeding,

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antibiotic exposure, and contact with caregivers [2]. This early microbial imprinting is crucial for the development of mucosal immunity, and may impact susceptibility to respiratory infections and allergic diseases later in life.

Functionally, the upper respiratory tract biofilm microbiota provides colonization resistance against pathogens by competing for adhesion sites and nutrients, producing antimicrobial peptides (e.g., bacteriocins), and modulating the local immune response. For instance, *Corynebacterium accolens* inhibits the growth of *Streptococcus pneumoniae* by releasing free fatty acids from host-derived triacylglycerols [3]. Similarly, *Dolosigranulum pigrum* has been associated with a reduced risk of otitis media and shows synergistic interactions with *Corynebacterium* spp. that promote mucosal health [4].

Changes in the qualitative composition of human microbiota due to increasing urbanisation, uncontrolled medicinal substance application, the use of air conditioners, aerosols, pasteurised products, and much more, lead to changes and, in some cases, to the formation of a biofilm on the surface of the mucous membranes of the upper respiratory tract, with individual characteristics: a specific species composition, in particular the ratio of pathogenic to non-pathogenic biota, coverage area, thickness, and degree of adhesion [5].

Rural inhabitants often have a more diverse microbiota composition, including bacteria associated with the environment and domestic animals (for example, representatives of the genera *Clostridia, Ruminococcus, Bacteroides*). This trend is usually observed due to more frequent contact with soil, domestic and farm animals, and the consumption of more natural products, particularly prebiotics, which leads to an increased concentration of postbiotics [6]. People in rural areas tend to have bacteria that stimulate the immune system, whereas urban residents generally exhibit lower species diversity. Dust in rural homes is richer in bacteria which promote the development of tolerance, while the urban environment is characterised by a more nature-isolated set of microorganisms and a higher content of allergens in household dust in urban dwellings [7].

Disruptions to the upper respiratory tract microbiota can lead to dysbiosis, reducing microbial diversity and enabling the overgrowth of opportunistic pathogens. Such alterations are linked to increased risk of acute and chronic conditions, including sinusitis, tonsillitis, otitis media, and even lower respiratory tract infections, especially in vulnerable populations [8]. Furthermore, emerging evidence suggests that the composition of the upper respiratory tract microbiota may influence systemic outcomes, including the progression of asthma and allergic rhinitis [9].

Within biofilms, microbial cells exhibit up to 1,000-fold increased tolerance to antibiotics compared to planktonic forms, contributing to chronic infections and recurrent disease episodes [10]. A major challenge in treating upper respiratory tract infections is antibiotic resistance, which is exacerbated by biofilm-associated persistence mechanisms [11]. For instance, *H. influenzae* and *M. catarrhalis* can survive high doses of β-lactam antibiotics due to altered metabolic states and the production of β-lactamases within biofilms [12]. Similarly, *S. aureus* biofilms exhibit increased resistance to fluoroquinolones and macrolides, driven by reduced antibiotic penetration and efflux pump activity. These adaptations reduce treatment efficacy and promote the selection of multidrug-resistant strains, particularly in settings of suboptimal antibiotic dosing or frequent treatment courses [13].

In addition to genetic resistance, biofilms create physical and biochemical barriers that limit the diffusion of antibiotics. The extracellular polymeric substance matrix can bind and inactivate antimicrobial agents, while creating gradients of oxygen, pH, and nutrients that slow bacterial metabolism and reduce drug susceptibility [14]. This is particularly problematic in the nasal and sinus cavities, where poor mucosal perfusion and anatomical complexity hinder drug delivery. Standard systemic antibiotic therapies often fail to achieve sufficient concentrations within biofilms to eradicate the microbial community. Given these limitations, nasal and sinus irrigation therapy (the mechanical lavage of the mucosa using saline or medicated solutions) has gained attention as an adjunctive treatment for biofilm-associated upper respiratory tract infections. Irrigation physically disrupts biofilms, clears mucus and debris, and improves mucociliary clearance, thereby reducing bacterial load and enhancing mucosal healing [15]. When combined with agents such as surfactants, antiseptics, or natural mineral waters, irrigation can also increase antimicrobial efficacy and reduce inflammation, while minimizing systemic side effects [16].

Objective

The aim of the study is to evaluate the *in vitro* effects of hydrogen carbonate sodium borine mineral water, alone and in combination with ciprofloxacin, on biofilm production intensity and structure by individual microbiota species of the upper respiratory tract mucous membrane.

MATERIALS AND METHOD

The study was performed on the basis of the State Non-profit Enterprise, Ukrainian Research Institute of Rehabilitation and Resort Therapy of the Ministry of Health of Ukraine in Odesa, Ukraine and the Scientific Centre of Marine Biology and Biotechnology and the Department of Microbiology, Virology and Biotechnology of the I. I. Mechnikov National University in Odesa. The study was conducted within the framework 6 Strategies and Programmess, State Non-profit Enterprise, Ukrainian Research Institute of Rehabilitation and Resort Therapy of the Ministry of Health of Ukraine in Odesa.

Research material. Microorganisms used in the study were: *Lactobacillus sporogenes*, isolated from the probiotic preparation (Mepro Pharmaceuticals Pvt. Ltd, UK), as well as strains obtained from the Department of Microbiology, Virology and Biotechnology microorganism collection (I. I. Mechnikov Odesa National University): *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923.

Previously, the *Lactobacillus* sp. was grown on De Man, Rogosa and Sharpe Agar medium, *E. coli* and *S. aureus* were cultivated on nutrient agar for 24 hrs at 37°C. The media used contained all the necessary nutrients for the bacterial development and their secondary metabolite production. The working microbial cell suspensions were standardized to $1.5 \cdot 10^8$ CFU/ml according to McFarland.

Ciprofloxacin (Ciprofloxacinum, OOO "Yuriya-Pharm", Ukraine) was used in the study at the minimum inhibitory concentration (MIC) previously determined by the serial dilution method in the 0.0156–2.0 mg/ml concentration range. Calculation of the results to determine the MIC value was carried out according to the recommendations of the European Committee on the determination of susceptibility to antibacterial agents (EUCAST). For *E. coli* the MIC was 0.0625 mg/ml, while this parameter for *S. aureus* was equal to 0.125 mg/ml. The sensitivity of the microbial strain obtained from the 'Lactovit Forte' probiotic determined that the MIC was 0.0625 mg/ml.

The factor of elimination-irrigation therapy, for which the level of antimicrobial activity was determined, was taken as sodium bicarbonate, boric, medium mineralization highly carbonated mineral water. Biofilm formation was assessed using standard 1% crystal violet solution staining and quantitative spectrophotometric measurement of optical density at 582 nm (OD_{582}) in 96-well plates during co-cultivation of *L. sporogenes* and opportunistic bacterium with: (1) control medium, (2) mineral water, (3) Ciprofloxacin alone, (4) mineral water in combination with Ciprofloxacin. The scheme of the study with mineral water treatment corresponds to a previous experiment [17]. To determine specific features of studied multispecies biofilm the detection of monospecies association formation was also carried out. The exposure of a sterile sample of mineral water was carried out within 10 min after the start of 4-hr bacterial cultivation, after which the microorganisms were poured with a fresh medium (in the Ciprofloxacin presence/absence) and the cultivation continued.

Light microscopy was performed using a Zeiss Primo Star light microscope (Germany) with a magnification of x1000 was used to analyze the structural architecture of mature biofilms of the studied strains. Photography was performed using a Canon EOS 750D camera (Japan) and Zen 2 software (ZEISS Efficient Navigation). The studied biofilms were characterized with corresponding parameters: area (μm^2), perimeter (μm), coverage (%), circularity (p.u.), aspect ratio (p.u.), number of biofilm regions (abs. u.) [18].

Statistical analysis. Each experiment was carried out in 3 repetitions; the number of repetitions in each case was equal to 10. The Student's t-test was used for comparative analysis of research results by Microsoft Excel 2013. A difference of $p \leq 0.05$ was considered significant.

RESULTS AND DISCUSSION.

According to a previous study, interactions between *L. sporogenes* and *E. coli* ATCC 25922 or *S. aureus* ATCC 25923 were detected [17], carried out both in the absence or presence of antibiotic with/without mineral water addition (Tab. 1). As a component of general human microbiome, corresponding microorganisms form associations characterized by close interactions: from antagonism to synergism, depending on the living conditions. Antibiotic therapy is one of factors that can influence the forms of interaction, especially if there is local antimicrobial drug application [11]. In a previous study by the authors, Ciprofloxacim provoked the transformation from antagonism into synergism (for *L. sporogenes* and *E. coli* ATCC 25922 interaction) and into neutralism (in the case of *L. sporogenes* and *S. aureus* ATCC 25923 co-cultivation). The treatment with mineral water restored interaction between the normal microbiota species and opportunistic microorganism in the presence of the studied antibiotic.

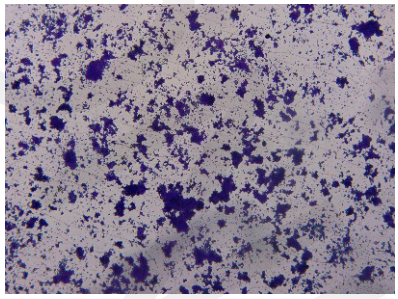
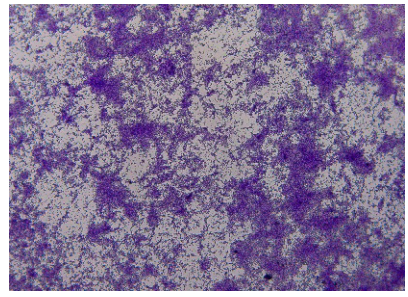
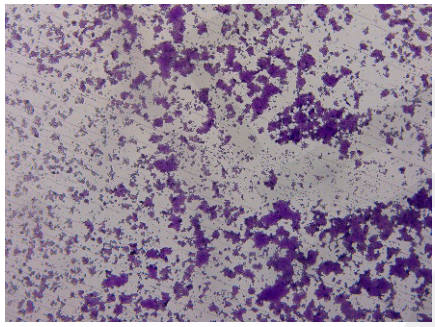
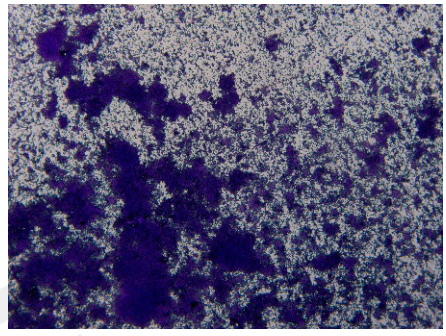
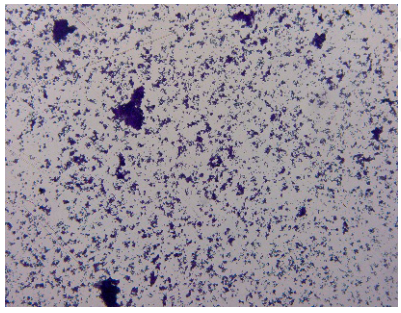
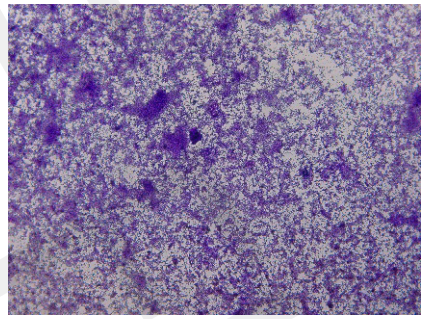
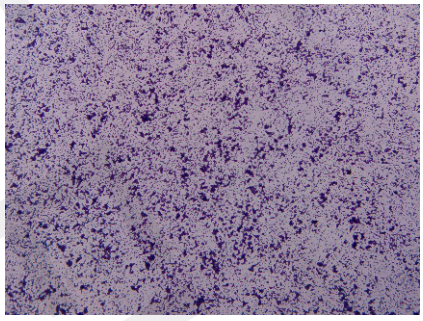
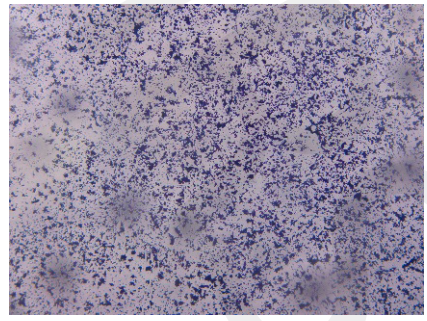
Due to the development of microorganisms in human microbiome mostly as a member of multispecies biofilm, not only influence on the form of interaction is one key component of effective antibiotic or irrigation therapy strategy [10] (Tab. 1).

Also, there is a need to detect changes of microbial association 3D-structure. Mineral water irrigation therapy is a promising noninvasive strategy for physically disrupting biofilms, reducing microbial load, and enhancing antibiotic efficacy [15]. This study is needed to optimize irrigation protocols and assess the long-term effects of such interventions on the microbiome and resistance. Based on the study data, there a characterization and comparison was obtained of the multispecies biofilm architecture influenced by Ciprofloxacin and the studied mineral water (Tab. 2).

Key morphometric parameters analyzed included biofilm area, perimeter, coverage, circularity, aspect ratio, and number of discrete biofilm regions (so-called microcolonies), which reflect both the compactness and spatial organization of biofilm association [19]. Describing these biofilm morphological parameters is essential in understanding the structure, maturation, and response of biofilms to environmental or antimicrobial factors. These metrics are typically quantified from 2D microscopic images, especially after staining methods (e.g. crystal violet). Biofilm area corresponds to total 2D surface occupied by biofilm in the image and indicates microbial biomass accumulation or extent of colonization. Thus, a larger biofilm area is associated with more mature biofilm or greater surface coverage [20]. Biofilm perimeter means the total length of the boundary enclosing the biofilm region, and reflects surface irregularity of microbial consortium. A high perimeter relative to area may indicate fragmented or rough biofilm structure [21]. Coverage (biofilm-covered area, %) is an image area covered by stained biofilm, a value indicating active adherence and exocellular matrix production by microbial cells [18]. Circularity is a ratio between biofilm area and its perimeter, a dimensionless shape factor ranging from 0 – 1. Lower circularity values are associated with dispersed or invasive biofilms, at the same time higher values suggest compact microcolonies in their contents [22]. Aspect ratio is a ratio of width to height of the minimum bounding rectangle or fitted ellipse. High aspect ratio indicates elongated or filamentous structures of biofilm microcolonies. This characteristic is dimensionless and if its value is calculated near 1.0, that suggests rounded colonies [23]. The number of biofilm regions corresponds with the amount of biofilm microcolonies. A large number of discrete colonies suggests early or fragmented biofilm development, or strain-specific patterning, depending on the microbial species [24].

The structural dynamics of multispecies biofilms composed of *L. sporogenes* co-cultured with either *E. coli* ATCC 25922 or *S. aureus* ATCC 25923 were assessed under 4 conditions: Control (no Ciprofloxacin, no Mineral Water); Mineral Water only; Ciprofloxacin only, and Ciprofloxacin with Mineral Water treatment. In both multispecies associations, Ciprofloxacin alone led to a significant increase in total biofilm area and coverage, particularly in *L. sporogenes* and *S. aureus* ATCC 25923 consortia (90.54×10^4 μm^2, 73.7%

Table 1. Microorganism biofilm structure and interaction form during growth *in vitro*

Microorganism	In the absence of Ciprofloxacin	In the presence of Ciprofloxacin, 0.0313 mg/ml
	Without mineral water treatment	
Lactobacillus sporogenes + *Escherichia coli* ATCC 25922	 Antagonism	 Synergism
Lactobacillus sporogenes + *Staphylococcus aureus* ATCC 25923	 Antagonism	 Neutralism
	With mineral water treatment	
Lactobacillus sporogenes + *Escherichia coli* ATCC 25922	 Antagonism	 Antagonism
Lactobacillus sporogenes + *Staphylococcus aureus* ATCC 25923	 Antagonism	 Antagonism

coverage). This may be linked to stress-induced biofilm promotion, a well-documented survival strategy in bacterial communities exposed to sub-lethal antibiotic concentrations. Antibiotics like Ciprofloxacin can trigger SOS-response and overproduction of exopolysaccharides, enhancing adherence and microcolony formation [19].

The decreased circularity and stable or high aspect ratio of the studied biofilms under Ciprofloxacin correspond to their more elongated or irregular structures, potentially due to filamentous cell morphology or channel formation. In the current study, it was found that mineral water is characterized by the possibility to modulate microbial biofilm

Table 2. Microorganism biofilm structure during growth *in vitro*

Biofilm parameter	Biofilm formation conditions			
	Control*	MW**	CF≠	(CF + MW)&
L. sporogenes + *E. coli* ATCC 25922				
Area, ×10⁴ μm²	28.25	14.99	56.62	36.71
Perimeter, μm	27.6	22.1	26.6	22.4
Coverage, %	23.0	12.2	46.1	29.9
Circularity, p.u.	0.37	0.36	0.26	0.22
Aspect ratio, p.u.	1.05	1.08	1.11	1.11
Number of biofilm regions, abs. u.	3350	4393	6298	9459
L. sporogenes + *S. aureus* ATCC 25923				
Area, ×10⁴ μm²	30.77	20.46	90.54	16.96
Perimeter, μm	22.2	17.0	32.3	21.9
Coverage, %	25.0	16.7	73.7	13.8
Circularity, p.u.	0.21	0.39	0.27	0.37
Aspect ratio, p.u.	1.21	1.11	1.04	1.03
Number of biofilm regions, abs. u.	4517	10153	2394	6086

Control* – no Ciprofloxacin, no Mineral Water; MW** – Mineral Water only; CF≠ – Ciprofloxacin only; (CF + MW)& – Ciprofloxacin with Mineral Water treatment; p.u. – per-unit; abs. u. – absolute units

structure, in particular its fragmentation and compactness. Also, there were different effects depending on bacterial species association. In the case of *L. sporogenes* and *E. coli* ATCC 25922 biofilm, mineral water alone reduced biofilm area and coverage (14.99×10⁴ μm², 12.2%) and increased the number of biofilm regions (4393 vs. 3350), indicating an intensive fragmentation process and less cohesive biofilm development. In contrast, during *L. sporogenes* and *S. aureus* ATCC 25923 co-cultivated biofilm, mineral water caused a 4.5-fold increase in the number of regions (10153 vs. 4517), with more circular and compact microcolonies: circularity increased from 0.21 to 0.39). These changes likely reflect ionic modulation of microbial surface charge and activity of *quorum sensing* system. Certain mineral components (e.g., Mg^2, Ca^2) can interfere with the biofilm matrix assembly and signal molecule diffusion, weakening interspecies synergy or enhancing detachment [16].

The next step of the study was the detection of the influence of the combination of Ciprofloxacin and mineral water on the biofilm development. Mixed effects of such a combination was demonstrated, depending on the type of microbial association. In the case of *L. sporogenes* and *E. coli* ATCC 25922 biofilm co-cultivation, the combined antibiotic/mineral water treatment reduced both biofilm parameters: area and circularity compared to Ciprofloxacin alone (36.71×10⁴ μm² vs. 56.62×10⁴ μm²). At the same time, these factors increased the number of discrete microcolonies to 9,459. This suggests structural destabilization, possibly due to mineral water disrupting EPS-induced consolidation. Conversely, in *L. sporogenes* and *S. aureus* ATCC 25923 multispecies biofilm, the area drastically dropped from 90.54×10⁴ μm² to 16.96×10⁴ μm² when mineral water was added to Ciprofloxacin, indicating a synergistic antibiofilm effect, possibly via enhanced membrane permeability or interference with matrix synthesis [25].

Recently, so-called heatmaps, which are quite powerful and visual tools for data visualization, have been increasingly used for the analysis of experimental data during microbiological studies, When constructing heatmaps, the numerical values of the results are replaced by colours to represent the magnitude of the results, which facilitates interpretation of the data. A heatmap was constructed in the current study to visualize the degree of influence of individual factors (antibiotics, mineral water, biofilm composition) on the main parameters of the studied multispecies biofilm architecture: area, μm²; circularity, p. u.; coverage, %; number of biofilm microcolonies. The constructed heatmap used a spectrum of colours corresponding to different values of influence: a lighter colour intensity was characterized by a smaller degree of influence on the corresponding parameter, while a more saturated (darker) colour – by a stronger influence. Values were the actual measurements for each condition-biofilm pair (Fig. 1).

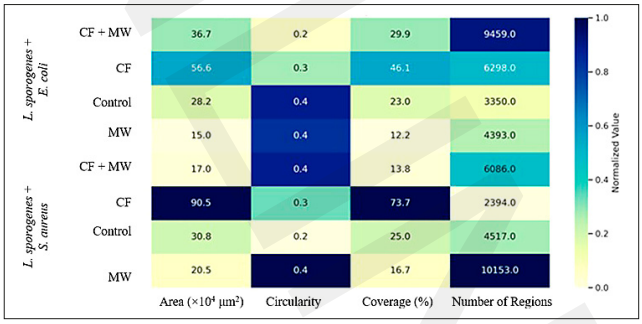


Figure 1. Heatmap of the studied biofilm parameters by different conditions and consortium.
Control – no Ciprofloxacin, no Mineral Water; MW – Mineral Water only; CF – Ciprofloxacin only; (CF + MW) – Ciprofloxacin with Mineral Water treatment

According to analysis of heatmap data, the following data were obtained:

- During *L. sporogenes* and *E. coli* biofilm co-cultivation, a moderate biofilm area (28.25×10⁴ μm²) with approximately 23% area coverage and intermediate circularity (0.37) was detected. The number of biofilm microcolonies (3,350) indicated a moderately cohesive biofilm. For *L. sporogenes* and *S. aureus* association area (30.77×10⁴ μm²) and area coverage (25%) were moderate; low circularity (0.21) meant irregular clusters; the microcolony number (4,517) was relatively high, indicating a heterogeneous discontinuous biofilm.
- In the case of *L. sporogenes* and *E. coli* co-cultivation, mineral water multispecies biofilm treatment caused a strong reduction in area (14.99×10⁴ μm²) and coverage (12.2%). Circularity remained stable (0.36), but the microcolony number increased (4,393), meaning that the biofilm became fragmented and less cohesive. For *L. sporogenes* and *S. aureus,* decreases were also detected *in* area (20.46×10⁴ μm²) and coverage (16.7%), but circularity (0.39) and microcolony amount in particular, more than doubled (10,153). Therefore, mineral water caused destabilization of the biofilm by promoting fragmentation into more circular, compact colonies, reducing overall biomass but increasing dispersion.
- The addition of Ciprofloxacin to the studied multispecies biofilms resulted in a significant increase in such parameters as area and coverage – nearly double compared to control for *L. sporogenes* and *E. coli* co-cultivation (56.62×10⁴ μm², 46.1%, respectively), and approximately in 3 times for *L. sporogenes* and *S. aureus* (90.54×10⁴ μm² of area and 73.7% of coverage). The other 2 characteristics of the

biofilm structure demonstrated opposite tendencies: for co-cultivation with Gram-negative bacteria, circularity dropped (0.26) and microcolony amount increased (6298), while for the Gram-positive species, the opposite applied – circularity improved (0.27) and microcolony number decreased (2,394). Thus, Ciprofloxacin triggered larger, irregular stress-driven *L. sporogenes* and *E. coli* biofilm amplification, consistent with literature on antibiotic-induced persistence strategies. For *L. sporogenes* and *S. aureus* biofilm, Ciprofloxacin also induced a massive, cohesive formation of biofilm, although possibly dominated by the strong exopolysaccharide production of *S. aureus* under stress.

- Mineral water – antibiotic combined treatment of the studied multispecies biofilm-initiated area (16.96×10^4 μm²) and coverage (13.8%) declined compared to Ciprofloxacin alone (36.71×10^4 μm², 29.9%), while the microcolony number rose sharply (9,459) in the case of *L. sporogenes* and *E. coli*. Thus, the studied combination destabilized the biofilm structure**,** breaking it into numerous irregular clusters**.** Mineral water may 'counteract' Ciprofloxacin-induced aggregation via strong suppression of the antibiotic-induced biofilm overgrowth, resulting in smaller but still compact colonies.

Research into alternatives to antibiotic therapy represents a new strategy, given the urgency of combating resistance to antibiotics in various pathogenic and opportunistic microorganisms. This fact acts as a stimulus for scientific investigations aimed at inhibiting or reducing the growth of biofilms on mucous membranes, thereby decreasing the chronicity of diseases.

In conclusion, in this study, mineral water demonstrated potential antibiofilm synergy with ciprofloxacin – suppressing large cohesive biofilm structures – while alone it promoted fragmentation rather than biomass growth. This dual effect may be leveraged in strategies aiming to destabilize biofilms of pathogenic and opportunistic microorganisms, and could be applicable for the treatment corresponding diseases of people with different life and conditional style.

CONCLUSIONS

Ciprofloxacin enhances biofilm formation in both bacterial consortia. The stronger effect was detected for combination of *Lactobacillus sporogenes* and *Staphylococcus aureus*, where biofilm mass nearly tripled compared with control.

Mineral water treatment can generally reduce biomass and coverage of the studied biofilms, but increases their fragmentation, in particular, growth of a more compact but smaller microcolony number.

The combination of Ciprofloxacin and mineral water for irrigation therapy demonstrated a destabilizing effect on Ciprofloxacin-induced biofilm of both studied microbial consortia *in vivo*.

In the case of *Lactobacillus sporogenes* and *Escherichia coli* ATCC 25922, mixed biofilm yielded many small, irregular microcolonies under the influence of the combination of Ciprofloxacin and mineral water

During the multispecies biofilm formation by *Lactobacillus sporogenes* and *Staphylococcus aureus* ATCC 25923, the potential for synergistic biofilm control was detected indicated by the combination of mineral water with Ciprofloxacin that corresponded to the prevention biofilm expansion, producing smaller, dispersed colonies.

Prospects for further research. The presented dual treatment strategy could be relevant for developing non-antibiotic-enhanced antibiofilm therapies, especially for medical and environmental applications (especially in the treatment of rural residents) where multispecies biofilms pose a resilience challenge.

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