



Influence of the combination of mineral water and ciprofloxacin on the interaction form of individual representatives of the upper respiratory tract mucosa microbiota *in vitro*

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Abstract

Introduction and Objective. Elimination irrigation therapy has been proposed as a potential treatment for upper respiratory tract infections, particularly after the COVID-19 pandemic, due to its antiviral properties and mechanical cleansing effects. Additionally, a combination of mineral water with antibiotic therapy has shown to be effective in improving the course of clinical infection and positively impact the immune system, potentially enhancing the normal state of microbiota state. The aim of the study is to investigate the influence of elimination-irrigation therapy using a combination of mineral water and ciprofloxacin on the interaction form of individual microbiota species of the upper respiratory tract mucous membrane.

Materials and Methods. During the study, microbiological methods were used, such as microscopic, bacteriological and biofilm cultivation methods.

Investigating antagonistic interactions within these associations, and a general decrease in microbial colonization activity. During the multispecies biofilm formation by *L. sporogenes* and *S. aureus* ATCC 25923, as well as *L. sporogenes* and *E. coli* ATCC 25922, a mutual antagonistic effect was determined. The forms of microbial interaction in multispecies biofilm was changed in the presence of 0.0313 mg/ml Ciprofloxacin.

Conclusions. The 10-minute mineral water treatment and addition of 0.0313 mg/ml Ciprofloxacin enhanced the antagonistic interaction between *L. sporogenes* and *E. coli* ATCC 25922, as well as with *S. aureus* ATCC 25923.

Key words

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

INTRODUCTION

The normal microbiota of the upper respiratory tract mainly consists of such genera representatives as *Prevotella*, *Sphingomonas*, *Megasphaera*, *Veillonella*, *Pseudomonas*, *Acinetobacter*, *Staphylococcus*, *Streptococcus*, and *Fusobacterium* [1]. During some inflammatory respiratory tract diseases, damage to the mucous membrane is noted that leads to a violation of the adhesion of microorganisms that constitute the normal microbiota of the corresponding epitope [2, 3].

In this regard, potential approaches aimed at restoring

mucosal homeostasis, implemented through direct effects on the microbiota composition and/or immune response modulation, may be of value for the prevention and treatment of respiratory infections, including COVID-19 [4, 5]. Recently, due to the COVID-19 pandemic, elimination irrigation therapy (EIT) is increasingly being used to treat the disease itself, as well as its prevention. The EIT procedure is hygienic care of the nasal cavity by washing with isotonic, hypo- and hypertonic solutions [6]. EIT provides mechanical cleansing of the mucous membrane, pathogen elimination, mucociliary transport normalization that facilitates nasal breathing, leading to a decrease of factors affecting the mucous membrane (bacteria, allergens, triggers, etc.) [6, 7]. In addition, during the washing, massage of the mucous membrane of the nasal cavity and tissue of the hypertrophied pharyngeal tonsils is performed [6].

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The concentration of the solutions used is important in EIT. Thus, the application of hypo-, iso- and slightly hypertonic solutions quickly and significantly reduces the microbial antigen amount, thereby decreasing the microbial load on the nasal mucosa [8, 9]. It was noted that hypertonic solutions stimulate the osmotic flow of ions through the apical surface of epithelial cells, changing the rheological properties of mucus, and reducing the fluid content of the mucous membrane that improves nasal breathing. The ability of marine water hypertonic solutions to stimulate the production of lysozyme, interferons, immunoglobulins, as well as to induce mucociliary transport, was indicated [9].

However, there are data on the possible negative ciliotoxic effect of hypertonic solutions, and the degree of its severity correlates with the concentration of the solution. In addition, hypertonic solutions have a pronounced irritating effect on the nasal mucosa, and are worse tolerated by patients than isotonic solutions [8]. The use of iso- and hypotonic salt solutions, for example, mineral water, reduces the ability of *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Staphylococcus haemolyticus*, *Streptococcus pneumoniae*, *Pseudomonas aeruginosa*, and *Moraxella catarrhalis* biofilm formation. At the same time, the study found that a hypotonic saline solution completely prevented the formation of biofilms in 84% of strains, while a hypertonic solution prevented only 38% of clinically isolated strains [8]. The scientific research about the amount of EIT directly impacting on the microbiota composition, both its qualitative and quantitative characteristics, is quite small, but today such a direction is becoming more and more relevant [2, 4].

Among the ready-made solutions that can be used for EIT, mineral waters are increasingly being considered. This is due to the fact that it has a unique mineral composition, a lower concentration of salts, unlike sea water, and a lower probability of side-effects. Due to this, it is often used for various diseases, and in prevention and rehabilitation therapy [8, 10]. The composition of mineral waters includes ions, more often K^+ , Ca^{2+} , Mg^{2+} , Na^+ , Cl^- , organic compounds (bitumen, phenols, volatile fatty acids, naphthenic and humic acids, fulvic acids), as well as its own microbiota. Together, these components can endow mineral waters with bactericidal properties [8], which is in contrast to marine water and hypertonic solutions, characterized by mechanical and physicochemical action, the direct suppressive effect of mineral waters on some opportunistic microorganisms is added [10].

The study was approved by the Institutional Commission on Bioethics (Protocol No. 3, 3 February 2023), and performed within the framework of funding by the Ministry of Health of Ukraine for the budgetary research work: 'Development of differentiated personalized complexes for the rehabilitation of patients after the transferred coronavirus infection at the sanatorium-resort stage' (State Registration No. 0122U001261).

OBJECTIVE

The aim of the work was to study the influence of elimination-irrigation therapy using a combination of mineral water and ciprofloxacin on the interaction of individual microbiota species on the upper respiratory tract mucous membrane.

MATERIALS AND METHOD

The study was carried out on the basis of the Ukrainian Research Institute of Medical Rehabilitation and Resort Therapy of the Ministry of Health of 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 Odessa, Ukraine.

Microorganisms used in the study included *Lactobacillus sporogenes*, isolated from the 'Laktovit Forte' probiotic preparation (Mepro Pharmaceuticals Private Limited, UK), as well as strains obtained from the Department of Microbiology, Virology and Biotechnology microorganism collection (I. I. Mechnikov Odesa National University, Odessa, Ukraine) – *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923.

Previously, the *Lactobacillus* sp. was grown for 24 h at a temperature of 37°C on De Man, Rogosa and Sharpe Agar medium. Nutrient agar was used for the of 24-hr *E. coli* and *S. aureus* cultures. The used media contained all the necessary nutrients for the bacterial development and their secondary metabolite production [11]. The working microbial cell suspensions were standardized to $1.5 \cdot 10^8$ CFU/ml, according to McFarland.

The minimum inhibitory concentration (MIC) of Ciprofloxacin (Ciprofloxacinum, YURiA-PHARM Corporation, Ukraine) was previously determined by the serial dilutions method. The Ciprofloxacin concentration range was 0.0156 – 2.0 mg/ml. Calculation of the results to determine the MIC value was carried out according to the European Committee on the determination of susceptibility to antibacterial agents (EUCAST) recommendations [12].

The EIT factor, for which the level of antimicrobial activity was determined, was taken from therapeutic table sodium bicarbonate, boric, medium mineralization highly carbonated water 'Polyana Kvasova Lagidna' (Ukraine), poured from well No. 1-sk (Tab. 1). The total mineralization of this mineral water was 6.62 g/l.

To determine the *Lactobacillus* sp. and opportunistic microorganism interaction forms, their cultivation was carried out during 24 hrs at 37°C:

- I. experiment variant – *L. sporogenes*, *E. coli* ATCC 25922 or *S. aureus* ATCC 25923 monocultivation;
- II. experiment variant – *L. sporogenes* and opportunistic bacterium co-cultivation.

At the same time, two series of studies were carried out in parallel; during the first, the cultivation of microorganisms was without Ciprofloxacin being adding to the medium; during the second – in its presence.

The scheme of the study with mineral water treatment corresponded with the previous experiment. Exposure of the mineral water sterile sample was carried out within 10 min after the 4-h bacterial cultivation, after which the microorganisms were poured with a fresh medium (in the presence/absence of Ciprofloxacin), and the cultivation was continued.

To determine the biofilm biomass after cultivation, its colouring was carried out according to the usual method using a 1% gentian violet aqueous solution. Optical density (OD) values were measured at 582 nm [13].

Table 1. Chemical composition of 'Polyana Kvasova Lagidna', 0.5 L; glass

Characteristics	CONCENTRATION, g/l	
	Value of characteristics according to condition requirements*	Composition of studied mineral water samples
Macrocomponents		
Sodium and potassium, g/l	1.3000–2.3000	1.7288
Calcium, g/l	<0.1500	0.0730
Magnesium, g/l	<0.0500	0.0191
Chlorides, g/l	<0.2000	0.1430
Sulfates, g/l	<0.0500	0.0115
Hydrocarbons, g/l	3.6000–6.3000	4.6462
Total mineralization, g/l	5.0000–9.0000	6.6216
Specific biological active components and substances		
Iodides, mg/l	≥5.00	0.23
Bromides, mg/l	≥25.00	0.54
Total iron, mg/l	≥10.00	<0.10
Orthoboric acid, mg/l	50.00–200.00	129.13
Metasilicic acid, mg/l	≥50.00	26.98
Total organic carbon, mg/l	5.00–30.00	<0.90

*according to *Mineral Healing Waters. Specifications: Industry Standard of Ukraine 42.10–02–96*. Ministry of Health, Kiev 1996, p. 30 (in Ukrainian). Order of the Ministry of Health of Ukraine, No. 243, dated 09/06/2003, concerning approval of the procedure for carrying out medico-biological assessment of the quality and value of natural medicinal resources, determination of methods of their use (in Ukrainian). Available from: <https://zakon.rada.gov.ua/laws/show/z0752–03#Text>.

The microbial interaction forms were determined according to [14]. Based on the obtained results, it was possible to establish the cell amount after co-cultivation, and also by comparing with the monoculture ones to note the form of interaction. The neutral microbial interaction form was detected if the OD values after co-cultivation (OD_{mix}) did not differ from the OD sum of the individual strains ($OD_{mix} = (OD_{L. sporogenes} + OD_{E.coli \text{ or } S. aureus})$). With the OD_{mix} predominance ($OD_{mix} > (OD_{L. sporogenes} + OD_{E.coli \text{ or } S. aureus})$) microbial synergism was detected. However, if the value OD_{mix} was less than the OD sum of the individual strains ($OD_{mix} < (OD_{L. sporogenes} + OD_{E.coli \text{ or } S. aureus})$), the antagonistic interaction form was determined.

In each experiment with mineral water, the control (sterile distilled aqua in a corresponding amount) was used to prevent and detect the 'mechanical' effect of solution influence. Each experiment was carried out in 3 repetitions; the number of repetitions in each was equal to 10. The Student's t-test was used for comparative analysis of research results. A difference of $p \leq 0.05$ was considered significant.

RESULTS

Recently, a large number of studies about the upper respiratory tract microbiota composition correction during the development of infectious diseases have increasingly focused on the application of traditional medicine [2, 15]. However, the use of systemic antimicrobials, in particular antibiotics, is still quite common which, in turn, can lead to a general microbiota disturbance: hypercolonization of mucous membranes by opportunistic microorganisms, such as *S. aureus* or *E. coli*, and there is also an increase of the bacterial persistent potential, in particular, the ability to form a biofilm [14, 15].

The first stage of the study detected that the MIC value of Ciprofloxacin, due to the studied microorganisms, differed quite significantly. According to the European Food Safety Agency, both representatives of the normobiota and those microorganisms that are part of probiotics used for the correction of dysbiosis, should not have any hereditary mechanisms of antibiotic resistance [16]. For *E. coli*, the MIC was 0.0625 mg/ml that indicates a fairly high level of sensitivity of this microorganism to the substance. As for *S. aureus*, the MIC 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.

At the second stage of the study, the characteristics of microorganism biofilm growth *in vitro* were determined (Tab. 2). It was been detected that the studied microorganisms, during monocultivation in the nutrient medium, practically did not react to the presence in it of Ciprofloxacin. Thus, the differences in the OD values that were fixed during comparing both experiment variants, did not exceed 10%, which caused their unreliability. The obtained results obviously indicate microbial resistance to the Ciprofloxacin action at the appropriate concentration.

In the human organism, as well as in the environment in general, unlike *in vitro*, microorganisms create associations in which they have close interactions at quite different forms: from antagonism to synergism, depending on the living conditions [14]. During the studied microorganism cultivation in the form of a multispecies association, their interaction forms were observed (Tab. 2). At the *L. sporogenes* and *S. aureus* multibiofilm formation, an antagonistic effect was observed: the mutual inhibition corresponded to almost 30% in comparison with the corresponding characteristics of monospecies associations. It is known that the ability of lactobacilli to suppress *S. aureus* growth is due to the lipase production, while other investigations detected that the inhibition mechanism during the multispecies association development is based on the release of molecules that suppress the expression of two genes involved in biofilm synthesis (*icaA* and *icaR*) [14].

Table 2. Microorganism biofilm growth characteristics *in vitro**

Microorganism	In the absence Ciprofloxacin	In the presence of Ciprofloxacin 0.0313 mg/ml
Monospecies cultivation		
<i>L. sporogenes</i>	0.287±0.016	0.269±0.012
<i>E. coli</i> ATCC 25922	0.298±0.022	0.311±0.015
<i>S. aureus</i> ATCC 25923	0.305±0.020	0.317±0.014
Multispecies cultivation		
<i>L. sporogenes</i> + <i>E. coli</i> ATCC 25922	0.514±0.012 [#]	0.920±0.024 [#]
<i>L. sporogenes</i> + <i>S. aureus</i> ATCC 25923	0.432±0.010 [#]	0.618±0.019

* results presented in the form of the OD value at 582 nm; # – difference is significant according to the corresponding sum of monospecies biofilm OD values formed in the antibiotic absence/presence.

neutral antagonistic synergistic form of interaction

A similar effect, but less expressed, was also noted during the *Lactobacillus* sp. and *E. coli* co-cultivation. Apparently, in this case, *E. coli* cells were susceptible to *L. sporogenes* signalling molecules that inhibited association formation [14].

At the formation of a multispecies biofilm in the presence of Ciprofloxacin, the following features were observed. First of

all, *Lactobacillus* sp. and *E. coli* interaction was characterized by a synergistic effect. The sum of the monospecies biomass ODs was lower than the corresponding ones of the multispecies biofilm. Thus, in this case, there was a change of interaction form, from antagonistic to synergistic, under the influence of Ciprofloxacin. There was also a change in the interaction between *L. sporogenes* and *S. aureus*: from antagonism to neutralism, determined by the absence of reliably significant differences in the corresponding OD values of mono- and multispecies biofilms.

Thus, the studied antibiotic changed the activity form of *L. sporogenes* against to *E. coli* and *S. aureus* cells. Perhaps this can be due to the fact that in the antibiotic presence with concentrations lower than the MIC values, the formation of microbial resistance occurs, one of the manifestations of which is the acceleration of cell division, and therefore enhancing the rate of surface colonization [15, 17]. The presence of Ciprofloxacin in the nutrient medium leads to the transformation of antagonism into neutralism (for *L. sporogenes* and *S. aureus*), or even synergism (for *L. sporogenes* and *E. coli*). This situation indicates that the antibiotic molecules can stimulate the surface colonization or block some regulation mechanism links during biofilm development [14]. This, therefore, can lead to the opposite result regarding the bactericidal effect of the antibiotic: the process of biofilm formation will accelerate, causing bacterial hypercolonization of the biotope, in particular, the upper respiratory tract mucous membrane [2].

At the next stage of the study, the effect of one of the elimination-irrigation therapy components, in particular mineral water, on the biofilm formation, both during mono- and multispecies cultivation, was investigated. Firstly, it was indicated that there was no differences between microbial biofilm growth in the presence of the studied mineral water sample and distilled water (a control, data not shown). Because of this fact, it was decided to compare the influence of the EIT components on bacterial development (Tab. 3).

The obtained results indicated that mineral water was a rather effective factor influencing the biofilm formation reducing the intensity of surface colonization by the studied microorganisms. In particular, the results obtained for the probiotic strain significantly differed from those detected for monobiofilm that developed without any additional factor (Tab. 3). For multispecies biofilms, a decrease was observed of their cell numbers under the mineral water treatment

Table 3. Characteristics of the growth of Microorganism biofilm under mineral water treatment*

MICROORGANISM	+MW	+(AB+MW)
Monospecies cultivation		
<i>L. sporogenes</i>	0.243±0.009 [#]	0.235±0.010 [#]
<i>E. coli</i> ATCC 25922	0.302±0.011	0.238±0.010 [#]
<i>S. aureus</i> ATCC 25923	0.295±0.008	0.213±0.011 [#]
Multispecies cultivation		
<i>L. sporogenes</i> + <i>E. coli</i> ATCC 25922	0.443±0.014 [#]	0.324±0.013 [#]
<i>L. sporogenes</i> + <i>S. aureus</i> ATCC 25923	0.405±0.012	0.400±0.012 [#]

* results presented as OD values; +MW – mineral water treatment of the biofilm formed in the antibiotic absence; +(AB+MW) – mineral water treatment of the biofilm formed in the antibiotic presence; # difference significant according to the corresponding OD values of mono-/polyspecies biofilm formed in the antibiotic absence (results shown in Tab. 2 and 3); & – difference is significant according to the corresponding OD values of mono-/polyspecies biofilm formed in the antibiotic presence (results shown in Tab. 2 and 3).

antagonistic form of microbial interaction

only in the case of *L. sporogenes* and *E. coli* co-cultivation. The decrease of the corresponding OD value was almost 15%, compared with that variant in the study, in which the multispecies biofilm formation was without additional factors.

The treatment of the biofilm with mineral water led to a significant inhibition of the development of any microbial associations on the surface.

DISCUSSION

For all studied monospecies biofilms a significant decrease of the cell amount was detected, compared with the data obtained from a similar experiment, but without additional treatment, in particular without either an antibiotic or mineral water. Obviously, the biofilm exposure by a mineral water hypotonic solution led to the inhibition of the surface colonization due to the removal of such substances as bacterial adhesins, or the neutralization of the microbial cell surface charge, which is necessary for the first stages of biofilm formation, by such components of mineral water as Na⁺ [18].

For multispecies biofilm, mutual inhibition of microorganisms in their composition was also observed. Compared with the OD value sums for separate cultivation of bacteria (under the same processing conditions), the OD values in this case were significantly lower. This corresponds to the antagonistic activity between the cells that formed the biofilm.

Comparing the data obtained in this study with those obtained for multispecies biofilms, but only under the Ciprofloxacin influence, a more expressed inhibitory effect was also observed. The difference between the corresponding OD values is about 20% for *L. sporogenes* with any opportunistic strain.

The mineral water composition included ions, more often K⁺, Ca²⁺, Mg²⁺, Na⁺, Cl⁻, organic compounds (bitumen, phenols, naphthenic, humic, fulvic and other acids) and its own microbiota. Na⁺ and Cl⁻ ions have a positive effect on the integrity of epithelial cells [18, 19]. Magnesium promotes cell repair and limits inflammation by reducing eicosanoid metabolism, both at the level of arachidonic acid release and through direct inhibition of 5-lipoxygenase [7]. Potassium is characterized by an anti-inflammatory effect [9]. Bicarbonate ions reduce mucus viscosity, although the relevance of adding pure bicarbonate to saline solutions is still debated [10]. According to research, boron and its derivatives have antiseptic, antibacterial and antifungal effects [20].

Together, all of the above ions significantly increase the viability of respiratory cells in contrast to isotonic physiological solution. Together, these components determine the bactericidal properties of mineral waters. Thus, in contrast to sea water and hypertonic solutions, in the case of mineral water treatment, to the mechanical and physicochemical action is added a direct suppressive effect on opportunistic microorganisms.

Therefore, additional treatment of biofilms, both mono- and multispecies, with an antibiotic and mineral water, provokes antagonistic interactions inside these associations and a general decrease in microbial colonization activity.

CONCLUSIONS

Biomass accumulation of *Lactobacillus sporogenes*, *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 did not change in the presence of 0.0313 mg/ml Ciprofloxacin during the biofilm formation *in vitro*.

During the multispecies biofilm formation by *Lactobacillus sporogenes* and *Staphylococcus aureus* ATCC 25923, as well as *Lactobacillus sporogenes* and *Escherichia coli* ATCC 25922, a mutual antagonistic effect was determined.

During the formation of a multispecies biofilm in the presence of 0.0313 mg/ml Ciprofloxacin, the form of cell interaction between *Lactobacillus sporogenes* and *Escherichia coli* ATCC 25922 was synergistic, while between *Lactobacillus sporogenes* and *Staphylococcus aureus* ATCC 25923, it was neutral.

The formation of both mono- and multispecies biofilms by the studied microorganisms was inhibited under the additional 10-minute treatment with mineral water. The 10-minute mineral water treatment and addition of 0.0313 mg/ml Ciprofloxacin enhanced the antagonistic interaction between *Lactobacillus sporogenes* and *Escherichia coli* ATCC 25922, as well as with *Staphylococcus aureus* ATCC 25923.

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