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Antibacterial and antibiofilm effects of radioactive thermal water

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Abstract. – OBJECTIVE: Most of the infections of the upper respiratory tract are caused by biofilm-forming microorganisms belonging to the *Pseudomonas*, *Streptococcus*, *Staphylococcus*, and *Enterobacter* genus. Many of these microorganisms also show antibiotic resistance, partly related to biofilm formation. The treatment of these affections may include inhalation of radioactive thermal water (RTW). The present study aimed to evaluate the *in vitro* antibiofilm effect of RTW collected from Merano springs, Italy.

MATERIALS AND METHODS: A series of experiments were performed evaluating the effect of RTW against planktonic cultures (1 h exposure) and on biofilms (10 min and 1 h exposure) formed by *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Pseudomonas aeruginosa*, and *Escherichia coli*. Viable biomass was assessed using a colorimetric assay. A model based on the infection by the same strains of a reconstituted human respiratory epithelium (RHRE) was used to morphologically evaluate the antibiofilm effect of RTW.

RESULTS: RTW decreased the viability of *S. aureus* and *S. pneumoniae* planktonic cultures by about 20%. RTW also decreased biofilm viability by all strains except for *E. coli* at both time points. In the RHRE model, bacterial adherence and colonization occurred in all specimens, showing a particular affinity for the epithelium's cilia. Bacterial infections caused significant alterations in the epithelium structure, showing enlargement of the intercellular spaces, and damage to the cell structure. Specimens infected with *S. aureus* showed slightly lower colonization levels after RTW treatment.

CONCLUSIONS: Results of this *in vitro* study showed a significant effect of RTW against Gram-positive planktonic bacterial cells as well as a significant antibiofilm activity.

Key Words:
Thermal water, Respiratory tract infections, Biofilms, Reconstituted epithelium, *Staphylococcus aureus, Streptococcus pneumoniae, Pseudomonas aeruginosa, Escherichia coli.*

Introduction

Respiratory tract infections are a significant public health issue, especially in older subjects, being a major cause of morbidity. Most of these infections are related to the upper respiratory tract, and the most common species involved belong to the *Pseudomonas, Streptococcus, Staphylococcus,* and *Enterobacter* genus[1-5]. Many of these microorganisms are resistant to antibiotics commonly used in general practice, such as ampicillin, amoxicillin, or kanamycin[6,7]. This issue represents a limit to the treatment options of such infections[8,9] and promotes the research of alternative therapies.

Most of these microorganisms are also biofilm-forming; biofilm formation may stably colonize the epithelium of the respiratory tract, leading to chronic and recurrent infections[4,10-12]. Biofilm organization allows implementing many strategies, including intercellular communication (quorum sensing), that contribute to enhanced antimicrobial resistance[10]. Cellular structures such as flagella, pili, and extracellular matrix are fundamental in the adherence and in initial colonization, making species such as *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* particularly fit for developing such chronic infections[13]. Furthermore, planktonic microbial cells are released from biofilms as a consequence

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of a dissemination activity, allowing further colonization. Consequently, biofilms can act as nuclei of infection that can create new metastatic nests of infection[14], thus playing a crucial role in the pathogenesis of recurrent respiratory tract infections.

^{222}Rn is a decay product of ^{238}U with a relatively short half-life (about 91 h); ^{222}Rn is brought to the earth surface by several sources, including thermal waters, and significantly contributes to human radiation exposur[15-18]. The radioactivity of this element is based on the generation of α particles that have a very high kinetic energy and low penetration capabilities, allowing it to act upon contact with tissues and biofilms[19].

Thermal springs located in Merano, Italy, represent a source of radioactivity that was reported to reach 481 Bq/l[20,21]. The treatment of chronic affections of the upper respiratory tract, both in adults and children, often includes inhalation of radioactive thermal water (RTW)[20,22-24]. However, the therapeutic mechanism of this type of treatment is not fully understood. ^{222}Rn is known to enhance antioxidant activity and suppresses the immune response in the human body[25,26]. Its activity could be due to its interaction with the lipidic components of the eukaryotic cellular membrane. It was suggested that ^{222}Rn could also interact with the lipids of bacterial cell walls, possibly affecting their growth[26]. Currently, there are no guidelines regarding the use of RTW for the treatment of biofilm-associated chronic respiratory tract infections[27].

The present study aimed to evaluate the antibiofilm effect of Merano RTW *in vitro*. The null hypothesis was that RTW does not show significant differences in the antibiofilm effect when compared to mains water (MW).

Materials and Methods

Collection of Water Samples

The tested RTW was collected in pyrex glass bottles by flushing without turbulence, avoiding the formation of air bubbles. Thermal water was tested within four hours after sampling when 97% of residual activity was present to minimize radon loss by decay. The measured activity of ^{222}Rn at the collection source was equal to 2050 Bq/l, and the residual activity of the tested thermal water corresponded to 1988.5 Bq/l. The antibiofilm effect of the RTW was compared with that of water collected from the mains immediately before the experiments. The composition of both RTW and MW is shown in Table I. All water samples were filter-sterilized (Millipore filter, 0.2 μm). A preliminary experiment was performed to exclude an influence of the filtration procedures on the residual radioactivity of RTW.

Microbiological Procedures

Culture media and reagents were purchased from Becton-Dickinson (BD Diagnostics-Difco, Franklin Lakes, NJ, USA). Tissue-culture treated multiwell polystyrene plates (Nunc 96-Well, 48-Well, and 24-Well) were purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA). *Staphylococcus aureus* ATCC 29213, *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922, and a strain of *Streptococcus pneumoniae* isolated from a patient suffering from upper and lower respiratory tract infection were grown on Columbia Agar supplemented with 5% sheep blood. The plates were incubated at 37°C in a 5% CO_2 atmosphere for 48 h. A suspension of each microorganism was obtained in Brain Heart Infusion (BHI) after overnight incubation at 37°C in a 5% CO_2 atmosphere. The bacterial cells were collected by centrifugation (2200 rpm, 19°C, 5 min), washed twice in phosphate-buffered saline (PBS), and resuspended in the same buffer. Each suspension was sonicated to disperse the bacterial aggregates (Sonifier model B-15, Branson, Danbury, CT, USA, 7W for 30 s), and

Table I. Chemical analysis of the control water (water from the means).

Constituent	
pH	7.6
Total dissolved solids	384 mg/L
Hardness	31°F
Electrical conductivity	591 μS/cm, 20°C
Ca	83 mg/L
Mg	18.9 mg/L
NH_4	< 0.10 mg/L
Cl	34 mg/L
SO_4	54 mg/L
K	2 mg/L
Na	18 mg/L
AS	< 2 μg/L
CO_3	234 mg/L
F	< 0.5 mg/L
NO_3	31 mg/L
NO_2	< 0.20 mg/L
Mn	< 1 μg/L

adjusted to 0.3 optical density units (OD) using a spectrophotometer at 550 nm (Genesys 10-S, ThermoSpectronic, Rochester, NY, USA). This value corresponds to an approximate microbial concentration of 6.00×10^8 cells/mL.

Evaluation of RTW Effect on Bacterial Cells in the Planktonic Phase

For this test, a total of 500 µl of each bacterial suspension and 500 µl of water were added to each well of 48-well plates. A total of 32 wells were used for each microorganism; 16 wells were incubated with RTW water while the other half (n = 16) were incubated with MW. The plates were incubated at 37°C in a 5% CO_2 atmosphere for one hour. In each well inoculated with thermal water, a dose of about 1 Bq was provided to the bacterial cells in one hour, reaching about 3600 disintegrations. Subsequently, the evaluation of the viable bacterial cells was performed using a colorimetric test based on the reduction of a tetrazolium salt (MTT assay).

Evaluation of RTW Effect on Biofilms

For this test, a total of 20 µl of each bacterial suspension and 180 µl of sterile BHI were placed into each well of 96-well plates. Sixty-four wells were used for each microorganism. The plates were incubated at 37°C in a 5% CO_2 atmosphere for 24 h to develop a multilayer biofilm. Subsequently, the culture medium was removed by gentle aspiration, and the wells were washed three times with 100 µl of sterile PBS. A total of 200 µl of each filter-sterile water was added to the wells (RTW, n = 32, and MW, n = 32). The plates were then incubated for an additional 10 min (n = 16) or one hour (n = 16) under the same conditions previously described.

In each well inoculated with RTW, a dose of about 0.4 Bq was provided in 10 min or one hour, reaching approximately 240 and 1440 disintegrations, respectively. At the end of the incubation period, the suspension was removed from the wells by gentle aspiration. The wells were then carefully washed three times with sterile PBS in order to remove non-adherent cells. After that, the evaluation of the viable biomass was performed using the MTT assay.

This experiment was repeated after ten days using the same setup, exposing the biofilms to the water samples for one hour. Biofilms treated with RTW were therefore exposed to the effect of an estimated residual ^{222}Rn activity of about 16% of the initial one (318 Bq/l).

Evaluation of Viable Bacterial Cells

MTT assay was performed as previously described[28]. Briefly, two starter MTT stock solutions were prepared by dissolving 5 mg/mL 3-(4,5)-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide in sterile PBS, and 0.3 mg/mL of N-methylphenazinium methyl sulphate (PMS) in sterile PBS. The solutions were stored at 2°C in light-proof vials until the day of the experiment when a fresh measurement solution (FMS) was made by mixing 1 mL of MTT stock solution, 1 mL of PMS stock solution and 8 mL of sterile PBS. A lysing solution (LS) was prepared by dissolving 10% v/v of sodium dodecyl sulfate and 50% v/v of dimethylformamide in distilled water.

At the end of the incubation period, the plates underwent MTT assay for the evaluation of adherent, viable and metabolically active biomass as follows: 200 µl of FMS were pipetted into each well of the 48-well plates used for determination of the effect on planktonic cultures. A total of 100 µl of FMS were pipetted into each well of the 96-well plates used for the biofilm formation experiments. All plates were incubated at 37°C in light-proof conditions for one hour.

During incubation, electron transport across the microbial plasma membrane and, to a lesser extent, microbial redox systems converted the yellow MTT salt to insoluble purple formazan. The conversion was facilitated by the intermediate electron acceptor (PMS). After that, a total of 500 µl from each well of the 48-well plates was then transferred to new plates, and 500 µl of LS were added. In the 96-well plates, the unreacted FMS was gently removed from the wells by aspiration, and the formazan crystals were then dissolved by adding 100 µl of LS into each well. All plates were then incubated at room temperature in light-proof conditions for one hour. A total of 90 µl of suspension from each well were then transferred to new 96-well plates, and optical density (550 nm) was measured (Genesys 10-S).

Evaluation of RTW Effect on Biofilms Developed on Reconstituted Airway Epithelium Model

To simulate the clinical conditions as accurately as possible, a model of reconstituted human respiratory epithelium (RHRE, MucilAir™ plates, Epithelix Sàrl, Plan-les-Ouates, Genève, Suisse) was infected with the different strains and used for the evaluation of the effect of the RTW on the formation of biofilms.

A total of 30 specimens of RHRE (six specimens for each tested microorganism and six control specimens) were used for this experiment. Specimens were shipped in 24-well plates containing agarose-nutrient transport medium. Upon arrival in the laboratory, the bags containing the specimens were opened under a laminar flow hood. The specimens were extracted from the transport plates, and the agarose was removed. Then specimens were placed in 6-well plates with nutrient medium (RPMI-1640 medium, supplemented with 20.0% fetal bovine serum, 1.0% L-glutamine, and 1.0% penicillin/streptomycin). Before testing, the culture plates were incubated at 37°C in a 5% CO_2 atmosphere and saturated humidity overnight.

A modified drip-flow bioreactor (MDFR) was used for this study (Figure 1). The device is a modification of a commercially available Drip Flow Reactor (DFR 110, Biosurface Technologies, Bozeman, MT, USA). The modified design allowed the placement of customized trays on the bottom of the flow cells and the immersion of RHRE air-lifted coupons into the surrounding flowing nutrient medium[29,30]. All tubing and specimen-containing trays of MDFR were sterilized before the beginning of the experiment using a hydrogen peroxide-based sterilization system (Sterrad, ASP, Irvine, Ca, USA). By limiting the maximum temperature to 45°C, heat-related damage to the whole system is avoided. The MDFR was then assembled inside a sterile hood. The specimens were placed into five polytetrafluoro-ethylene (PTFE) trays containing six holes each, which fixed them and exposed their surfaces to the flow medium. All trays were fixed on the bottom of the flow chambers of the MDFR and immediately inoculated with fresh nutrient medium. The MDFR was transferred into an incubator operating at 37°C, 5% of CO_2, and saturated humidity atmosphere. Then, a multichannel, computer-controlled peristaltic pump (RP-1, Rainin, Emeryville, CA, USA) was turned on and used to provide a constant flow of nutrient medium through the flow cells (9.6 ml/h).

After 24 h, the pump was stopped, and a total of 100 µl of each bacterial suspension was pipetted on the surface of each of six specimens in the first four flow cells. The remaining flow cell was left as a negative control. The bioreactor was operated for an additional 24 h to allow the development of a multilayer biofilm on the surfaces of the RHRE. After that, the flow was stopped, the specimens carefully extracted, and three specimens for each group were exposed to each tested water (RTW or MW). After one hour, the morphological aspects of the specimens were evaluated using Scanning Electron Microscopy (SEM).

Morphological Analysis Using SEM

Specimens subjected to SEM analysis were gently rinsed three times with sterile PBS to remove non-adherent cells, then placed into a cacodylate-buffered 2% glutaraldehyde-fixative solution (pH = 7.4) for 48 h. The specimens were then passed through a graded ethanol series (50, 70, 80, 85, 90, 95, and 100%, v/v). Finally, the specimens were subjected to critical point drying (Critical-Point Dryer, EMS 850, Hatfield, PA, USA), mounted on stubs with conductive glue, sputter-coated (JEOL FFC-1100, Japan), and analyzed with a scanning electron microscope (JEOL

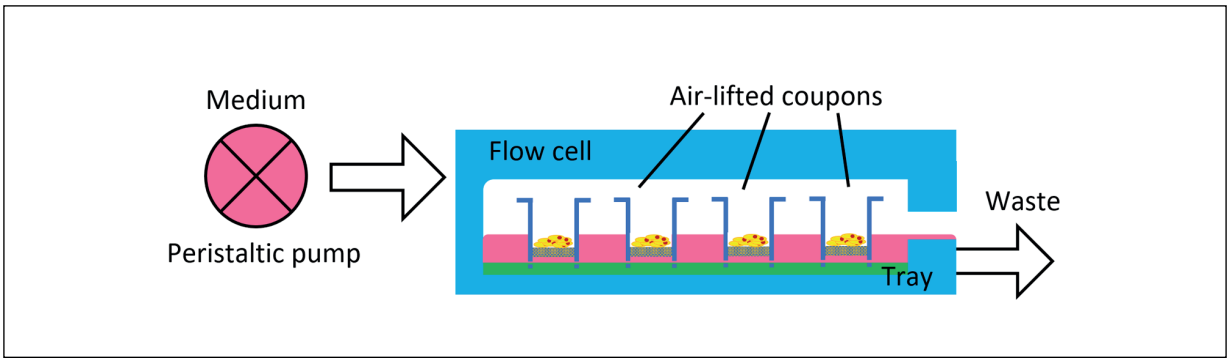

Figure 1. Functioning diagram of the RHRE specimen-bearing bioreactor. The peristaltic pump provides a constant flow of supplemented medium to the flow cells for the specified amount of time. It was possible to infect the specimens with the tested bacterial strains by removing the lid of the bioreactor under sterile conditions. In the same way the specimens could be exposed to the test or control water.

JSM-840A, Japan) at magnifications of 5000×-15000×. Four randomly selected fields were recorded for each specimen.

Statistical Analysis

Statistical analyses were performed using JMP 10.0 software (SAS Institute, Cary, NC, USA). Normal distribution of viable biomass data was checked using Shapiro-Wilk's test, and homogeneity of variances was verified using Levene's test. Means and standard errors were calculated from the raw data. Two-way analysis of variance (ANOVA) was performed considering the microorganism and the water as fixed factors. Tukey's honestly significant difference was used as a post-hoc test. The level of significance was set to $p<0.05$.

Results

Viable Biomass Assessment (MTT Assay)

The results regarding the effect of RTW on planktonic cells are shown in Figure 2A. Both factors showed a highly significant influence on cell viability, and no interaction was found between them. A significant decrease in cell viability after exposure to RTW was demonstrated for *S. aureus* and *S. pneumoniae* (24%, $p=0.002$, and 22%, $p=0.039$ compared to the MW control, respectively). No significant differences were found for the other two tested microorganisms.

The effect of RTW on biofilms after an exposure of 10 min and one hour is shown in Figure 2B and 2C, respectively. After a 10 min exposure, ANOVA showed that both factors significantly influenced biofilm formation, and there was a significant interaction between them ($p=0.022$). A significant decrease in cell viability after exposure to RTW was demonstrated for *S. aureus* and *S. pneumoniae* (13%, $p<0.001$, and 9%, $p=0.019$ compared to the MW control, respectively).

After one-hour exposure, ANOVA showed that both factors significantly influenced biofilm formation, and there was a significant interaction between them ($p<0.001$). A significant decrease in cell viability after exposure to RTW was demonstrated for *S. aureus, S. pneumoniae,* and *P. aeruginosa* (8%, $p<0.001$, 9%, $p=0.037$, and 18%, $p<0.001$ compared to the MW control, respectively).

The effect of 10 days-stored RTW on biofilms (one-hour exposure) is shown in Figure 2D. A significant influence of the microorganism factor was found ($p<0.001$), while the water factor

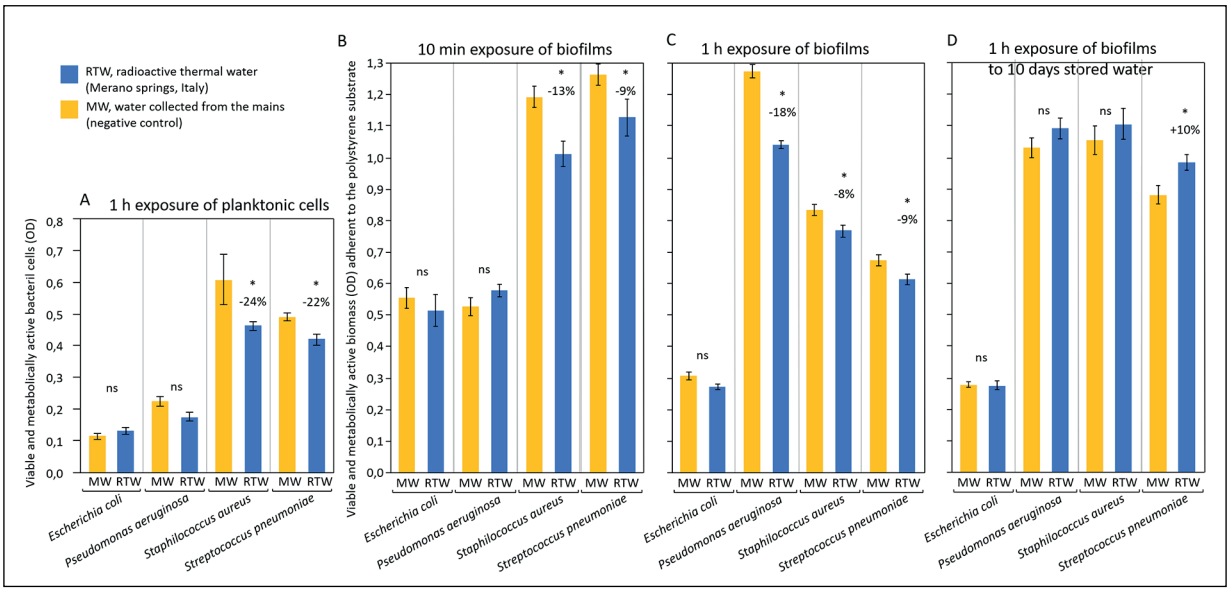

Figure 2. The results of the viable biomass assay are reported in the panel as means ± 1 SE. **A,** Evaluation of RTW effect on bacterial cells in the planktonic phase; **B,** Evaluation of RTW effect on biofilms after a 10-min exposure, or after a one-hour exposure **(C)**; **D,** Evaluation of the residual effect of a ten days-stored RTW on biofilms after a one-hour exposure. Results of the statistical analysis are reported as [ns] = non-significant differences were found between the tested and the control water ($p>0.05$), or [*, ±xx%] = a significant difference is highlighted, and the percentage of reduction or increase in bacterial viability of RTW-treated groups compared to the control is displayed.

was non-significant (p=0.072), and there was no interaction between the factors (p=0.442). A significant increase in cell viability after exposure to RTW was demonstrated for *S. pneumoniae* (10%, p=0.026, compared to the control).

Morphological Analysis (SEM)

The morphological aspects of the RHRE infected with the tested microorganisms and exposed to RTW and MW are shown in Figure 3. All observations demonstrated bacterial adherence and colonization, showing a particular affinity for the epithelium's cilia. The bacterial infection caused significant alterations in the epithelium structure, showing enlargement of the intercellular spaces, and damage to the cell structure itself. Specimens infected with *S. aureus* showed slightly lower colonization levels after treatment with RTW when compared to the MW control, in agreement with viable biomass data. No influences of RTW on the other microorganism colonization were found. Also, no influences of RTW were found on the non-infected RHRE specimens, all of them showing complete preservation of the anatomical features.

Discussion

The epithelium of the respiratory tract acts as a complex interface with the external environment, facing repeated challenges by pathogens as well as allergens and pollutants[20]. The infection of the respiratory tract is strictly related to biofilm-forming pathogenic microorganisms. This feature includes the confinement of bacteria to a specific location and the possibility to resist eradication by antibiotic therapy, in spite of the susceptibility of bacterial cells to the antibiotics while in the planktonic phase[10]. As a consequence, this phenomenon is often related to the chronicization of biofilm-associated infections. Treatments of chronic affections of the respira-

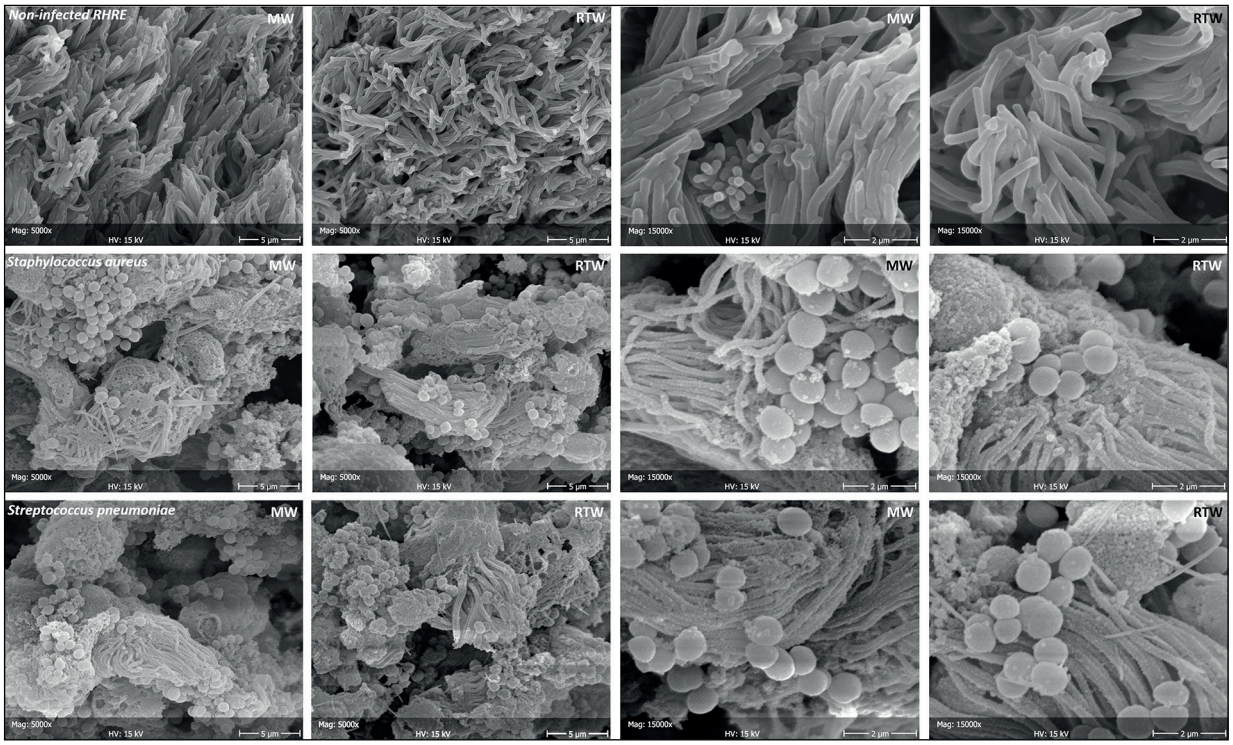

Figure 3. SEM analysis of the RHRE model. Non-infected epitheliums showed complete preservation of the anatomical features. No influences of the exposure to RTW were found on the non-infected RHRE specimens. Micrographs depicting the infection of the RHRE by the tested Gram-positive bacteria are displayed. All observations demonstrated bacterial adherence and colonization, showing a particular affinity for the epithelium's cilia. The bacterial infection caused significant alterations to the epithelium structure, showing enlargement of the intercellular spaces, and damage to the cell structure itself. Specimens infected with *S. aureus* showed slightly lower colonization levels after treatment with RTW water when compared to the MW control, in agreement with viable biomass data. No influences of RTW on the infection by the other tested microorganisms were found (micrographs not shown).

tory tract may include the inhalation of thermal water. However, no current guidelines exist due to uncertainty about the indications and action mechanisms[27].

Composition, properties, and possible indications of the thermal waters, including radioactive ones, are generally well known. Nevertheless, there is no data about the effect of RTW on biofilms and biofilm-associated respiratory tract infections.

Our results showed that the tested RTW was able to significantly reduce cell viability of planktonic cultures of some of the most common pathogens associated with respiratory tract infections. The reduction was strain-dependent, and the effect of the RTW was significant on Gram-positive bacteria (*S. aureus, S. pneumoniae*) but not on Gram-negative ones (*P. aeruginosa, E. coli*). The tested water also reduced biofilm viability by the same microorganisms on a standard polystyrene substrate after 10 min exposure. An extended exposure (one hour) also showed a reduction in the viability of *P. aeruginosa* biofilms. Therefore, the null hypothesis that the RTW does not show significant differences in the antibiofilm effect when compared to MW was rejected.

Interestingly, when the RTW was re-tested after a ten-day storage time, no reduction effect on biofilm viability was demonstrated. This latter experiment showed that the antibiofilm effect of the tested thermal water disappeared over time, suggesting a link with its radioactivity.

No effect of the RTW was seen on the reconstituted human respiratory epithelium. On the contrary, specimens infected with *S. aureus* showed a slightly lower biofilm formation after treatment with the RTW when compared to the control.

The possibility of growing reconstituted human respiratory epithelium *in vitro* is a very promising possibility that allows replicating clinical conditions in a realistic way. Studies on the efficacy of novel drugs or compounds whose effect on human tissues is unknown are just an example[31]. However, a disadvantage of this system is that specific conditions, such as allergic rhinitis or other chronic affections, still cannot be adequately replicated. In the present study, a model of bacterial infection of the respiratory tract was simulated by successfully developing biofilms on the RHRE. This model allowed to test the efficacy of a treatment in conditions as close as possible to the clinical ones. In this case, the detection methods could not rely on the same viable biomass assay (MTT), due to the response that would have come both from human cells as well as bacteria. It was therefore chosen to study bacterial interactions with the RHRE as well as the effect of the tested treatment on this model using a morphological approach (SEM). This approach showed the colonization of the epithelium by the tested bacterial strains, with a particular affinity for the epithelium's cilia, with a morphological aspect demonstrating high similarity to the clinical one. Bacterial colonization of the epithelium caused significant alterations in its structure, showing enlargement of the intercellular spaces, and damage to the cell structure itself.

The activity mechanism of ^{222}Rn on human respiratory tissues is not fully understood[26]. An enhancement in the antioxidant activity and suppression of the immune response was proposed and is commonly acknowledged[25,26]. The properties of this element, including its liposolubility, have been put into relation with its interactions with the lipidic components of the cell membrane and bacterial cell wall. Almost no information on the activity of ^{222}Rn on bacterial cells is available in the literature. Serrano et al[26] hypothesized that the interaction of this element with the lipids of the bacterial membrane could affect bacterial growth. Our results may provide additional insight since the effect of ^{222}Rn was only shown on Gram-positive planktonic cells. It is known that Gram-positive bacteria have a thick cell wall made of peptidoglycans; contrarily, Gram-negative cells have a much thinner structure. It may be speculated that a thicker cell wall may gather a higher amount of ^{222}Rn particles, which in turn express a higher activity on these cells compared to Gram-negative ones. In the literature, the effect of radiation on bacteria has been studied considering ^{239}Pu and ^{237}Np elements[19], that emit a similar type of radiation as ^{222}Rn (α particles). Contrarily to the other types of radiations that are highly penetrating and deposit their energy along their path in relation to the materials' density, α particles have little penetrating power. In this way, they cause a relatively high damage that is typically restricted to few tens of μm in tissues[19]. Reed et al[32] showed that an α particle emitter, ^{239}Pu, caused a higher loss in bacterial viability of *Chelatobacterheintzii* compared to ɣ radiation. The authors attributed this activity to the bioassociation of ^{239}Puwith the bacterial colonies. This mechanism could explain the effect of ^{222}Rn on biofilms that was highlighted in the present study.

The infected RHRE model allowed to evaluate the therapeutical effects of the tested RTW under controlled conditions, very similar to *in vivo* ones. Nevertheless, *in vivo* studies are still necessary as *in vitro* simulations can, no matter how accurately, reproduce only parts of the very complex interactions taking place in the human respiratory environment. It is a balancing act between the speed, cost, and purpose of the model that dictates its selection and use.

Conclusions

These results open the possibility to test an association of the effect of RTW with antibiotic therapies that are conventionally performed against biofilm-associated respiratory tract infections. The bacterial susceptibility to the antibiotic compounds may be modulated by this association, possibly increasing their efficacy and reducing side effects.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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