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Regenerative effects of spring water-derived bacterial lysates on human skin fibroblast in *in vitro* culture: preliminary results

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Abstract

Objective: Previous studies have shown regenerative power of the skin with Comano (Trento, Italy) spring water and resident non-pathogenic microflora. This study investigated the action of bacterial lysates that were isolated from Comano spring water on *in vitro* culture of human skin fibroblasts.

Methods: For this study, we selected the following four bacterial lysates: L1 (closest relative: *Rudaea cellulosilytica*), L2 (closest relative: *Mesorhizobium erdmanii*), L3 (closest relative: *Herbiconiux ginsengi*), and L4 (closest relative: *Fictibacillus phosphorivorans*). Human fibroblasts were cultured under Dulbecco's modified Eagle's medium (DMEM) with bacterial lysates added or DMEM (controls). Cell proliferation was evaluated by spectrophotometric absorbance analysis after the XTT-Microculture Tetrazolium Assay.

Results: At 24 hours, cultures with L2, L3, and L4 showed a higher absorbance compared with controls. At 48 hours, cultures with L1, L2, and L3 showed slightly lower absorbance compared with controls, and culture with L4 showed a higher absorbance than in the other experimental conditions. At 72 hours, absorbance was lower in cultures with L1, L2, and L3 than in controls, and absorbance was higher in culture with L4 than in the other experimental conditions.

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Conclusions: Our study indicates a favorable action of Comano spring water microbiota on proliferation of human skin fibroblasts.

Keywords
Regeneration, microbiota, spring water, bacterial lysate, cell culture, fibroblast

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Introduction

Comano (Trento, Italy) spring water is a hypotonic, bicarbonate calcium–magnesium mineral water with a well-recognized anti-inflammatory action. Comano spring water is specifically used for treating various inflammatory-based cutaneous disorders, such as psoriasis and atopic dermatitis, and in treating upper airway disorders, including allergic and vasomotor rhinitis, rhinosinusitis, pharyngitis, and laryngitis. The supposed mechanisms of action of this spring water are mucosal tissue stabilization, immune response modulation, pro-inflammatory agent scavenging, and increased mucociliary clearance.[1–5]

Besides the anti-inflammatory effects in Comano spring water, novel regenerative properties were recently demonstrated by our research group as follows. An experimental animal *in vivo* model showed increased proliferation and migration of keratinocytes, as well as favorable modulation of regeneration of dermal collagen and elastic fibers.[6] The latter finding was confirmed in an *in vitro* experimental model of cultured human skin fibroblasts.[7] We found that cells that were maintained in Dulbecco's modified Eagle's medium (DMEM) with 20% Comano spring water showed 31% higher vitality than did control cells that were cultured in DMEM alone. A further study on an *ex vivo* human skin wound model showed a reduction in inflammatory cell infiltration with selective fibroblast recruitment after application of Comano spring water.[8] These favorable cellular effects included increased neocollagen synthesis and regeneration of elastic fibers.

A native microbial community was recently described with a progressive degree of accuracy in Comano spring water.[9–11] Therefore, we hypothesized that the active metabolites produced by the spring water's microbiota are related to the regenerative effects reported above. In this study, we investigated the action of selected bacterial lysates that were isolated from Comano spring water on *in vitro* culture of human skin fibroblasts.

Materials and methods

The project was conducted in collaboration among the Plastic and Reconstructive Surgery Unit of the ICS Maugeri SB SpA IRCCS in Pavia (Italy), the Immunology and General Pathology Laboratory of the Department of Molecular Medicine of the University of Pavia (Italy), the Laboratory of Microbial Genomics of the Department of Cellular, Computational and Integrative Biology (CIBIO) of the University of Trento (Italy), and the G.B. Mattei Institute in Comano (Italy). The experiments were carried out from January 2018 to June 2018. The study was approved by the Ethics Committee of the ICS Maugeri

SB SpA IRCCS, Pavia (Italy) (project identification code, 2064) on 26 September 2016. The study conformed to the 1975 Declaration of Helsinki and informed written and signed consent was obtained from all of the patients.

Bacterial isolation

All water samples were collected in sterile bottles. Immersion samples were taken using singularly packed sterile bottles and handled with sterile forceps.

Water samples of 100 mL were concentrated by filtration using 0.22-μm Pall Supor filters (Pall Corporation, Port Washington, NY, USA). Filters were then placed on standard Petri dishes with Reasoner's 2A agar (S2Media, Spokane Valley, WA, USA), which is a medium used for enumeration of bacteria from potable water. Growth media were prepared using filtered Comano spring water to maintain the native chemical composition of this environment.

Bacterial identification and selection

Bacterial strains that were isolated from the Comano spring water were characterized by genome sequencing. The bacterial collection comprised 182 isolates.[11] The genome of the closest relative to each isolate was used to calculate the average nucleotide identity (ANI) using the software Pyani (Leighton Pritchard, The James Hutton Institute, Dundee, Scotland, UK).[12]

The bacterial collection was screened for anti-inflammatory activity (unpublished/undisclosed data) using an *in vitro* assay. Four isolates of the collection were then selected to assess their regenerative properties. We selected two Gram-positive and two Gram-negative isolates of which ANI values ranged between 80% and 90%, which indicated that they represent undescribed species from known genera. The selected isolates were as follows. Lysate 1 had an isolate code of 3F27F6 and an ANI value of 82.6%, with the closest relative of *Rudaea cellulosilytica* of the phylum Proteobacteria (L1). Lysate 2 had an isolate code of 3P27G6 and an ANI value of 86.5%, with the closest relative of *Mesorhizobium erdmanii* of the phylum Proteobacteria (L2). Lysate 3 had an isolate code of 3FA27C10 and an ANI value of 83.1%, with closest relative of *Herbiconiux ginseng* of the phylum Actinobacteria (L3). Lysate 4 had an isolate code of E and an ANI value of 89.8%, with the closest relative of *Fictibacillus phosphorivorans* of the phylum Firmicutes (L4).

Preparation of bacterial lysates

Bacteria were grown for 3 days at 27°C in R2A broth, pelleted in plastic tubes, washed in Dulbecco's phosphate-buffered saline (PBS) (Sigma-Aldrich, Catalog number D8537), and resuspended in Dulbecco's PBS to a final volume of approximately 1/60 of the initial volume. Bacterial cells were then sterilized and lysed by treatment in an autoclave at 121°C for 20 minutes. These cells were then mixed to resuspend the insoluble fraction, frozen immediately in liquid nitrogen to avoid insoluble fraction sedimentation, and stored at −20°C overnight. Samples were then lyophilized and pulverized by shaking in plastic tubes containing sterile glass beads. The plastic tubes containing the bacterial powders were stored at room temperature and protected from light.

Human skin specimen collection

Human skin samples were obtained from anatomical specimens that were harvested during sessions of reduction mammaplasty. These sessions were performed by the same surgeon in the same Surgical Unit at different times on four healthy female patients

with ages ranging from 43 to 60 years (43, 47, 51, and 60 years old). Each patient provided a single skin specimen that underwent the same standard experimental processing. The patients were consecutively enrolled according to the Unit's surgical activity schedule and there was no overlapping among skin fibroblast cultures that were derived from different patients.

The specimens were stored in sterile containers filled with sterile saline solution (S.A.L.F. SpA, Cenate Sotto, Bergamo, Italy) that was enriched with 1% (10,000 U/ml) penicillin and streptomycin (10 mg/ml) (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). The specimens were then transported in ice to the Immunology and General Pathology Laboratory for further processing. The time lag between tissue harvesting and the start of the laboratory procedures was approximately 15 minutes.

Human skin specimen processing

Human skin specimens were processed in a sterile environment with a second-class laminar flow hood. The specimens were placed on a sterile cutting board and adipose tissue was removed from the dermis using sterile tweezers and scissors.

Fibroblast cell cultures

Four small split thickness samples of dermis, sized 1×1 mm, were harvested from each specimen's deep dermal surface. The samples were placed into culture flasks (Sigma-Aldrich) and incubated in an atmosphere of 95% humidified air with 5% CO_2 at 37°C for 10 minutes to allow adhesion to the flask's surface. After this time, 6 mL of DMEM (DMEM powder with 4500 mg/L glucose, 0.584 g/L L-glutamine, and 0.11 g/L sodium pyruvate) (Sigma-Aldrich), which was reconstituted with distilled water (Milli-Q, Merck-Millipore, Darmstadt, Germany) and enriched with 3.7 g/L sodium bicarbonate, 10% fetal bovine serum, 1% (10,000 U/mL) penicillin, and streptomycin (10 mg/mL) (all from Sigma-Aldrich), was added to the flasks. Thereafter, the flasks were incubated in an atmosphere of 95% humidified air with 5% CO_2 at 37°C until sub-confluence was obtained. At this stage, the dermal samples and culture medium were removed, and the flasks were rinsed with PBS. After removal of PBS, 4 mL of trypsin were added. The flasks were incubated in a humidified atmosphere of 95% air with 5% CO_2 at 37°C for 5 to 6 minutes. An equal amount of DMEM was added to each flask and the cell suspension was aspirated and centrifuged at $306 \times g$ for 5 minutes at room temperature (Sigma Laboratory Centrifuge 4K15; DJB Labcare Ltd., Buckinghamshire, England). After centrifugation, the fluid component was discarded and the cell pellet was re-suspended with 2 mL of DMEM. At this stage, to measure the vital cell count, 10 μL of the cell suspension were added to 10 μL of 1:2 diluted trypan blue and this was placed in a Bürker chamber. A count of 2000 cells/100 μL was considered the threshold for progressing to the next step. The cells were then seeded in new flasks at a 10^4 cells and 5 mL of DMEM were added. The flasks were incubated in an atmosphere of 95% humidified air with 5% CO_2 at 37°C until sub-confluence was obtained. At this stage, the whole sequence was repeated twice up to sub-confluence. At the end of the last cycle, the cell suspension was seeded with a multichannel pipette in 15 wells within a 96-multiwell plate at a concentration of 2×10^3 cells. The plate was incubated in an atmosphere of 95% humidified air with 5% CO_2 at 37°C for 20 minutes to allow cell adhesion within the wells (Figure 1). After this time, the cultures were carried out under two experimental conditions of DMEM with bacterial lysates added and DMEM alone (controls).

In the cultures with addition of bacterial lysates, DMEM was removed and a delicate rinse with PBS was carried out. After

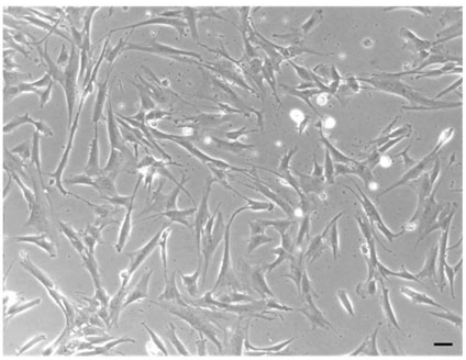

Figure 1. Human skin fibroblasts ready for culture under different experimental conditions (bar: 10 μm).

removal of PBS, 100 μL of a culture medium that comprised 99.67% DMEM and 0.33% bacterial lysate suspension were added. Four bacterial lysates were tested and each culture medium was tested in three wells to provide reliable spectrophotometric data. The bacterial lysate suspensions were made in distilled water at a concentration of 0.33%. In the control cultures, DMEM was topped up to an eventual 100 μL volume and cultures were carried out in three wells, similar to the experimental conditions.

All of the cultures were incubated in an atmosphere of 95% humidified air with 5% CO_2 at 37°C. After a further 24 hours, the culture media were removed from all of the wells and a delicate rinse was carried out with PBS. After removal of PBS, each well was refilled with 100 μL of the same culture medium (DMEM or DMEM with bacterial lysates added).

XTT-Microculture Tetrazolium Assay

Quantification of cell proliferation and viability was evaluated using spectrophotometric analysis to measure absorbance at 475 nm after cell staining with the XTT-Microculture Tetrazolium Assay using the SPECTROstar Omega microplate reader (BMG Labtech-Euroclone; Pero, Milano, Italy). Three multiwell plates without cells were filled with 100 μL of DMEM to provide a mean baseline "white" absorbance assessment. XTT solution was prepared by mixing 5 mL of XTT labeling reagent with 0.1 mL of electron coupling reagent. Within each well with cells, the medium was removed, and a delicate rinse was carried out with PBS. After removal of PBS, each well was refilled with 100 μL of DMEM. A volume of 50 μl of XTT solution was then added to each well and incubated in a humidified atmosphere of 95% air with 5% CO_2 at 37°C for 4 hours.

The first spectrophotometric analysis was carried out 24 hours after addition of the bacterial lysates (T_1) together with the white absorbance assessment. The XTT staining procedure was repeated at 48 hours after addition of the bacterial lysates (T_2). At this time, the culture media were removed from all of the wells that would continue the culture. A delicate rinse was carried out with PBS. After removal of PBS, each well was refilled with 100 μL of the same culture medium (DMEM or DMEM with bacterial lysates added). The XTT staining procedure was repeated again at 72 hours after addition of the bacterial lysates (T_3).

The mean absorbance (Abs) value in the wells for each experimental condition, including the white absorbance value, was calculated at 24, 48, and 72 hours. The actual absorbance value in every experimental condition at each time interval (n) was calculated according to the following formula:

$$\mathrm{Abs}_{n\,(475\ \mathrm{nm})} = \left(\frac{\sum Abs_n\ sample}{3} - \frac{\sum Abs\ white}{3}\right)$$

Each assay was performed in triplicate. Results are expressed as the mean ± standard deviation of the absorbance

Table 1. Absorbance values at different times in the different experimental conditions.

	Culture conditions				
Time	DMEM	DMEM + lysate 1	DMEM + lysate 2	DMEM + lysate 3	DMEM + lysate 4
24 hours	0.022 (0.009)	0.019 (0.013)	0.273 (0.284)	0.420 (0.223)	0.491 (0.123)
48 hours	0.766 (0.029)	0.686 (0.161)	0.750 (0.160)	0.737 (0.161)	0.840 (0.067)
72 hours	0.915 (0.079)	0.842 (0.059)	0.776 (0.057)	0.771 (0.044)	1.006 (0.055)

Footnote: Values are mean (standard deviation).
DMEM: Dulbecco's Modified Eagle's medium.
Lysate 1: Proteobacteria, Gammaproteobacteria, Xanthomonadales, Rhondobacteraceae, Rudaea sp.
Lysate 2: Proteobacteria, Alphaproteobacteria, Rhizobiales, Phyllobacteriaceae, Mesorhizobium sp.
Lysate 3: Actinobacteria, Microbacteriaceae.
Lysate 4: Firmicutes, Bacillaceae.

value in the different experimental conditions at the different times. Because of the limited size of the sample and that these were preliminary experiments, no formal statistical test (except for mean absorbance values and standard deviation) was applied to evaluate any significant variations at different times in the different experimental conditions.

Results

At 24 hours, mean absorbance was slightly lower in the culture with L1 than in controls (Table 1). All of the cultures with the other bacterial lysates showed a higher mean absorbance compared with controls and L1 culture, and there was a progressive ascending trend in absorbance values from the L2 to L4 cultures. The culture with L4 showed the highest mean absorbance at the time of observation.

At 48 hours, mean absorbance was slightly less in the cultures with L1, L2, and L3 than in controls. The culture with L1 showed the lowest mean absorbance. At this time, mean absorbance was higher in the culture with L4 than in all of the other experimental conditions.

At 72 hours, mean absorbance values were less in the cultures with L1, L2, and L3 than in controls. The culture with L3 showed the lowest absorbance. Mean absorbance was higher in the culture with L4 than in all of the other experimental conditions.

These absorbance results are also summarized in Figure 2.

Discussion

Recent studies have shown anti-inflammatory, immunoprotective, and regenerative properties in some spring waters and these properties are associated with active metabolites produced by the water's native non-pathogenic microflora.[6–9,13–17] The first evidence of such an association was reported in the spring water of La Roche-Posay (France) in which anti-inflammatory properties have long been recognized.[13–15] The lysate of *Vitreoscilla filiformis*, which is a Gram-negative aerobic bacterium within the Neisseriaceae family that was isolated in these spring waters, caused enhancement of the skin defense mechanisms through activation of cutaneous regulatory T cells.[16] A study in Avène (France) spring water showed an association between the water's anti-inflammatory properties and a biological extract from *Aquaphilus dolomiae*. This bacterium, which is from the Neisseriaceae family, has been isolated in

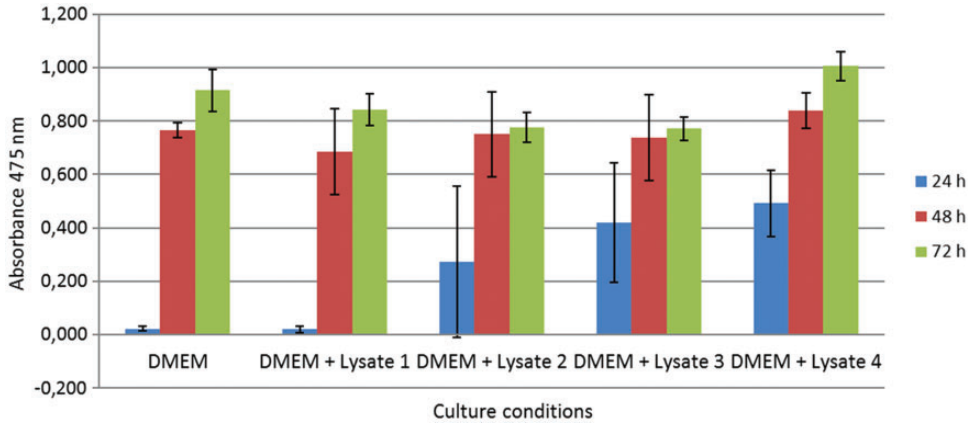

Figure 2. Mean absorbance values ± standard deviation at different times in the different experimental conditions.

the Avène spring water, and shows properties that enhance the innate cutaneous immune response by activation of Toll-like receptors.[17]

Previous investigations by our research group using different experimental models showed regenerative power of the skin by Comano spring water. Comano spring water promoted increased keratinocyte proliferation and migration, reduction of inflammatory cell infiltration, selective fibroblast recruitment, and favorable modulation of regeneration of dermal collagen and elastic fibers.[6–9] One of our studies also showed that 20% replacement of DMEM culture medium with filtered Comano spring water through a 0.2-µm porous membrane promoted higher proliferation of *in vitro* cultured human fibroblasts than in controls cultured with DMEM alone.[7]

Comano spring water is certified as bacteriologically pure, which means that it contains neither pathogenic microorganisms nor microorganisms with fecal or other contamination. However, our previous study showed a resident non-pathogenic microflora of which the composition displays seasonal variations.[9,18] The complete spring water microbiota was subsequently categorized using genomic sequencing techniques.[10,11] Interestingly, all of the bacteria that were isolated from the native microbiota in spring water belong to unknown bacterial genera that are likely to be specific to the Comano water source.

In the current study, we collected bacteria for lysing on the 3rd day of growth because we found that 3 days was an adequate time to achieve a sufficient amount of biomass to produce the lysates used in this study. We are aware that synthesis of bacterial products varies in identity and amount over the growth curve. Nevertheless, because the four selected species are not phylogenetically related, we decided that, in this preliminary screening phase, interspecies comparison of the different growth phases would not be meaningful. Further studies could be performed for each species separately to assess how the regenerative effect of bacterial lysates might vary as a function of the growth phase. We chose to test the regenerative properties of some of the most bioactive (anti-inflammatory) lysates derived from two Gram-negative and two Gram-positive bacteria. This is because the composition of the cell wall may affect

regenerative activity. On the basis of screening that was performed to assess anti-inflammatory activity of bacterial lysates, in the present study, we used bacterial powder at 0.33%. This concentration was chosen because higher concentrations had a negative effect on the anti-inflammatory assay for most lysates tested, and lower concentrations displayed a lower effect. None of the applied lysates demonstrated toxic effects on cultured fibroblasts. The small size of the sample only allowed descriptive statistic to be performed, which suggested some preliminary considerations.

Among the four bacterial species in our study, we observed interesting stimulation of cell proliferation only after addition of Firmicutes-derived bacterial lysates to the culture medium. L1 showed a weak inhibitory power on fibroblast growth throughout the observation time. L2 and L3 showed enhancement of cell proliferation, followed by a decrease in rate of proliferation by 72 hours, with an eventual cell proliferation rate that was lower than that in controls.

To date, little information is available on the genera *Bacillus*/*Exiguobacterium*/*Oceanobacillus* within the Bacillaceae family of the Firmicutes phylum. *Exiguobacterium* is involved in biodegradation of plastic as part of plastic-eating mealworms' gut microbiota.[19] The species in L4 that were isolated and tested in our study is unknown. Furthermore, stimulation of proliferation of fibroblasts is the first reported action on human cells attributable to this new bacterium. Interestingly, because L4 showed less anti-inflammatory action versus the other lysates in the pre-study selection, its stimulation of cell proliferation might be independent from an anti-inflammatory effect. Therefore, the mechanism of action of this bacterium in enhancing cell proliferation remains unknown. Therefore, further investigations need to be carried out to clearly define the detailed molecular mechanism of action of this bacterium in the skin regeneration process. A few reports have shown the role of some other microorganisms in enhancing the wound healing process through control of the inflammatory response.[20,21] There is an ever-growing interest in the role of microbiota not only in promoting tissue regeneration, but also as a source of novel therapeutic options.[22]

The assessment modality in the present study (XTT-Microculture Tetrazolium Assay with spectrophotometric analysis) is different from our previous study with the same experimental model using filtered spring water (direct vision microscopic cell count within a Bürker chamber).[7] Therefore, we are unable to make any objective comparison between earlier and current experiments. However, stimulation of fibroblast growth was generally consistent and thus somewhat comparable across studies.

The observation time in our study was restricted to 72 hours. At this time threshold, cultured cell proliferation reaches an environmental saturation point. Beyond this time frame, the experimental model's validity is compromised. The current study is considered as a preliminary step in a more complex ongoing trial. Our human *ex-vivo* skin culture model has already been proven effective in demonstrating a regenerative effect of Comano water on human skin.[8] This model is currently being used to assess any interaction of specific water-derived bacterial lysates with the human skin regeneration process. To optimize the major trial's cost/effectiveness ratio, we decided to preliminarily check the gross regenerative potential of selected bacterial lysates in a simple *in vitro* fibroblast culture model.

Conclusions

Our study shows a favorable action of the native microbiota in Comano spring water

on human fibroblast proliferation. This regenerative effect might not be associated only with the well-known anti-inflammatory action of spring water. A combination of biological properties of several bacterial species within this spring water might be responsible for its regenerative effects on human skin.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the author on reasonable request.

Authors' contributions

GN directed the trial and wrote the article.
MS performed the experiments on cell cultures.
MMT gathered the data and contributed to writing the article.
AF conceived the study and wrote the article.
MB performed isolation of the bacterial lysates.
MC directed the bacterial lysate research.

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Declaration of conflicting interest

The authors declare that there is no conflict of interest.

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