Viable microbial community composition of Agnano Thermal Spring Water

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Abstract

The Agnano thermal spring water (ATSW) is mainly a salso-bromo-iodic-bicarbonate-alkaline-sulphurous water. Since ancient time, the benefits of thermal spring water in the treatment of various diseases have been known. Today it is known that thermal spring water acts effectively on various physiological and pathological processes such as skin regeneration, respiratory and cardiovascular diseases and bone illnesses and most of these beneficial effects depend on the chemical and physical properties of water. However, all the benefits of thermal spring water may not be fully explained only by its mineral composition. In this regard, it is known that non-pathogenic bacterial populations play an active role in various processes in the ecological and biological fields. The aim of the present study is the microbiological characterization of ATSW through the use of two distinct methods in order to determine the total viable bacterial community.

Introduction

The Agnano thermal spring water (ATSW), located in the largest thermal basin in Italy with its 72 springs of various nature and characteristics, have been the best expression of this for millennia. Already in the first century. a.C., the Hellenists understood its high curative value, both physical and spiritual, later in the second century. d.C. the Romans began to build structures for the exploitation of these portentous waters. Indeed, with the allocation of the Romans in Flegrea Area began the real thermal activity across the Campi Flegrei. The impressive thermal spa of the Hadrian era (117-138 d.C.) which stands on the south-western side of the Agnano basin, on the slopes of Monte Spina, testifies that thermalism was widespread in Agnano. The benefits of thermal spring water in the treatment of various diseases have been known since ancient times. In fact, thermal spring water has therapeutic functions and can be used in the treatment of various dysfunctions, from respiratory and rheumatic diseases to gynecological and gastrointestinal diseases. Today it is known that thermal spring water acts effectively on various physiological and pathological processes such as: skin regeneration (Liang et al., 2015), cardiovascular diseases (Oyama et al., 2013) and bone diseases (Fioravanti et al., 2011) and most of these beneficial effects depend on the chemical and physical properties of water.

However, all the benefits of thermal spring water, such as improved migration and cell proliferation or anti-inflammatory and regenerative properties, may not be fully explained only by its mineral composition. In this regard, it is known that non-pathogenic bacterial populations play an active role in various processes in the ecological and biological fields (Nicoletti et al., 2015; Zeichner et al., 2018). Maintaining the physiological intestinal well-being is a clear example of how a bacterial microflora is essential for regulating homeostasis, metabolic functions and the immunological response (Sommer et al., 2017).

Sulfurous waters are the most numerous in Italy and the presence of H_2S influences immunological response (Valitutti et al., 1990) and have beneficial effects on neurodegenerative disorders, respiratory tract (Keller et al., 2014) and in particular on cardiac functions, as also carbonic waters.

Agnano's thermal baths belong to the largest volcanic area of the Mediterranean Sea (ca 400 Kmq). It is the only example of thermal spring water extended in urbane area with a huge impact landscape and a great archeological significance. The Agnano thermal spring water (ATSW) is mainly a salso-bromo-iodic-bicarbonate-alkaline-sulphurous water. Thanks to the variability of the temperature and the abundance of mineral elements (sodium chloride, iodine, bromine, calcium, bicarbonate, sulphides), a great number of different disorders can be treated (cardiovascular, gynecological, dermatological and rheumatic).

The aim of the present study is the microbiological characterization of ATSW through the use of two distinct methods in order to determine the total viable bacterial community.

Material and Methods

ATSW collection

ATSW was collected in two different seasons of the year; in winter (January 2019) and in autumn (October 2019) with an aseptic procedure. Using surgical gloves, 3000 ml of water was collected with a sterile 50 ml syringe, close to the thermal spring. The samples were poured into three 1 L containers for microbiological analysis (VWR) and transported as soon as possible for microbiological analysis and processed rapidly following collection. The *in situ* temperature of water, in winter's sampling, was 55.4 °C and *in situ* pH was 6.8 - 7 whereas, in autumn's sampling, the temperature was 56 °C and pH was still 6.8 - 7. Chemical composition of ATSW was reported in**Table 1** .

Microbiological analysis

Filtration procedure and isolation of bacteria

A portion of 100 ml of ATSW was filtered through seven 0.20 µm pore cellulose nitrate membranes (Nalgene 0.2 Analytical Filter Unit; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Each membrane was plated on differential and selective media (Tryptic Soy Agar Blood BD, Chocolate agar BD, McConkey agar Oxoid, Mannitol Salt agar Oxoid, Sabouraud dextrose agar Oxoid, Colistin Nalidixic acid agar Biolife, Schaedler agar BD) incubated in aerobic condition at 37°C for 2-3 days and in anaerobic condition at 37°C for 3 days. For autumn sampling, the bacterial plates were also incubated at 55 °C in order to mimic the temperature of thermal water. Microbial growth was then recorded and each pure culture was subsequently prepared and stored at -80° C. At the same times, 300 ml of water sample was filtered through 0.20 µm pore cellulose nitrate membrane and the filter was soaked in 5ml of 1X phosphate-buffered saline (PBS, containing 140mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4, 18mM KH_2PO_4, pH 7.4) in a centrifugal tube (Di Natale et al., 2018). Vortexing was performed for 2–3 min with a vortex touch mixer (HeidolphREAX 2000) to evaluate the presence of*Legionella pneumophila* antigen (ALERE BINAXNOW® Legionella antigen card) following the instructions of the manufacturer.

Enrichment procedure and isolation of bacteria

ATSW sample (10 ml) was transferred to nutrient Brain Hearth Infusion broth (90 ml) (Oxoid, Thermo Fisher Scientific, Inc., Waltham, MA, USA). The sample was incubated at 37°C with 220 rpm until turbidity was been detectable. For autumn sampling, the bacterial inocula were also incubated at 55 °C with 220 rpm. After the appropriate incubation, the broth culture was centrifugated at 3500 rpm for 10' at room temperature, and serial dilutions were plated on differential and selective media as mentioned above. Separated colonies were then obtained by streaking on selective agar media for several times.

Identification of bacterial isolates

The identification of all bacterial isolates was performed by mass spectrometry using the Matrix Assisted Laser Desorption/Ionization (MALDI) mass spectrometer (Bruker Daltonics, MALDI Biotyper, Fremont,

CA, USA), a high-throughput proteomic technique for identification of a variety of bacterial and fungal species (Sogawa K. et. al, 2011; Ferrazzano G.F. et. al, 2017; Cicatiello A.G. et al, 2014). Through this identification procedure a reliability threshold of 1.4 was established.

Results and Discussion

Microbiological analyses for the two collections of ATSW, "Nuove Terme di Agnano srl", was performed during the winter and autumn seasons through two procedures, in order to increase the yield for the microbiological characterization. The two procedures, filtration and enrichment, highlighted in both sampling the abundance of bacterial species belonging mainly to three different phyla: Actinobacteria, Firmicutes and Proteobacteria with a prevalence of species belonging to the phylum Firmicutes (Table 2). The filtration procedure determined the isolation of four bacterial isolates for winter sampling and ten isolates form autumn sampling, while the enrichment method revealed the presence in thermal water of sixteen different bacterial species for winter sampling and eight bacterial species for autumn sampling (Table 3). The analysis showed a difference between the bacterial populations associated with seasonal changes, highlighting a majority of species belonging to the genus *Staphylococcus* , with a prevalence of Coagulase-negative *Staphylococci* (ConS) for winter sampling using enrichment procedure, and the genus *Bacillus* for autumn sampling, using both procedures. To underline, the species *Pseudomonas stutzeri* and *Bacillus subtilis* have been isolated from cultures incubated at 50°C for autumn sampling.

The most represented phylum, Firmicutes, mainly includes gram-positive species with a genome characterized by a low GC content and includes several mobile and capable of forming endospores species (Schleifer, 2009). The members of this phylum are abundant in soil and water where they are involved in the decomposition and recycling of organic matter (Gibbons and Murray, 1978). However, different genera of phylum Firmicutes belong to the normal human flora or can be associated with diseases in humans and animals (Baik et al., 2008). Moreover, some members of this phylum may have an industrial use for the production of enzymes or antimicrobial and antifungal activities, such as the species*Lactobacillus plantarum* , which is used in the food industry, and*Bacillus pumilus* , which has an antimicrobial activity towards*Vibrio* spp. and fungicide (Liu et al., 2012) (both isolated during the autumn sampling by filtration procedure).

Within the phylum Proteobacteria, in particular, in the Gamma proteobacteria some *Pseudomonas* spp. like *P. luteola* and*P. stutzeri* , could be relevant because they are both involved in bio-absorption's mechanisms. *P. luteola* is involved in bio-absorption processes of heavy metals such as chromium and aluminum in acid environments, metal ions often present in industrial waste water and is also involved in the absorption of copper and nickel as it is a producer of exopolysaccharide (Ozdemir and Baysal, 2004). Also, the species *P. stutzeri* is considered an excellent candidate for the simultaneous absorption of nitrogen and phosphate in waste water, in fact it oxidizes ammonia exhibiting the ability of nitrification and denitrification as well as the ability to degrade organophosphorus pesticides (Ozdemir et al., 2005).

Among the identified phyla is represented, although to a lesser extent, the phylum Actinobacteria. To this phylum belong different genera of Gram positive bacteria present both in terrestrial and aquatic environments. These microorganisms have a great importance for the human being since agriculture and forests depend on the contribution of these bacteria to the processes that take place in the soil. In this environment, the Actinobacteria grow with characteristics similar to fungi, forming extensive structures similar to mycelia, favoring the decomposition of the organic matter of dead organisms so as to provide the essential elements for plant growth (Servin et al., 2008).

Therefore, the ATSW analyzed is rich in non-pathogenic bacteria that may play a role in treating various skin disease (Balato et al., 2019). As ATSW contains live bacteria that could impact the skin's microbiota, the water itself is considered to be a probiotic (Zeichner et al., 2018). Although probiotics have been widely used in the past for the treatment/prevention of gastrointestinal disorders, a growing number of evidences has suggested that they can modulate the composition of microbial community, exerting their healthy effect directly or indirectly on the skin (Balato et al., 2019; Zeichner et al., 2018).

Moreover, the ATSW is a rich source of minerals. About 31 minerals have been identified of which 17 are

found as major ions (>/= 0,4 mg/l) like chloride, sodium, bicarbonate, sulphate, carbon dioxide, potassium, silicon, calcium, magnesium, boron, ammonium, bromide, strontium, litium, fluoride, manganese, arsenic.

Sodium helps to maintain the suppleness of skin due to its water-holding capacity. Hence it is very useful in dry dermatoses like psoriasis and ichthyosis. It also helps in desquamation of hyperkeratotic lesions (Rivaz et al., 2011).

Noteworty, among cations of interest, Mg^{2+} and Ca^{2+} have been recognized for their benefits in skin barrier recovery (Denda et a., l999). In normal skin, magnesium and calcium ions were localized with a high concentration in the upper epidermis (Proksch et al., 2005). After barrier disruption, the gradients of calcium, magnesium, and potassium in the epidermis disappeared while the pH was not altered (Denda et a., l999). Loss in the ion gradient is a signal for an increase in proliferation, differentiation and lipid synthesis aimed to repair the perturbed barrier (Lee et al., 1992, Denda et al., 2003). Magnesium is also essential for cell metabolism. It helps in maturation and differentiation of keratinocytes. Hence it is very useful in psoriatic patients who have a low magnesium level in the serum and scales (Rivaz et al., 2011). Calcium-and magnesium-rich thermal spring waters are known to improve skin barrier function and accelerate wound healing (Proksch et al., 2005).

Moreover, the soothing and protective properties of thermal spring waters in sensitive skin (antioxidant or anti-ageing) are enhanced by the presence of trace elements such as selenium, strontium (Celerier et al., 1995). These properties have been demonstrated in many studies using human keratinocytes, fibroblasts or other response-appropriate cell lines (Seite et al., 2013; Joly et al., 1998). Furthermore, a recent study demonstrated the activity of salso-bromo-iodine water on mucous-secretory disorders, in particular it helped to improve the relationship between the mucous-protein complexes and the water: initially, this action was congestive, subsequently, it became an anti-catarrhal, anti-inflammatory, antiseptic and immunostimulant action (La Mantia et al., 2018).

The ATWS for its mineral composition and microbial diversity exhibits both prebiotic and probiotic characteristic, thus it will be analyzed for its anti-inflammatory and regenerative properties in an *in vitro* system.

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