EVIDENCE OF MICROBIAL ANTAGONISM IN VOLCANIC TUFF ROCK

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A total number of 64 bacterial strains isolated from two volcanic tuff rock located in Teisani, Prahova, and Malul Alb, Buzau areas were investigated for ability to producing factor with antagonistic activity (AF). From these, 59 strains have the ability to synthesis AF acting against other strains isolated from these ecosystems. Some of the strains (1–2 and 1–7 respectively, isolated from Malul Alb) are acting as antimicrobial against pathogenic strains *Staphyloccocus aureus* and *Lysteria monocytogenes*. Fourier Transform Infrared Spectroscopy (FTIR) and X-Ray fluorescence (XRF) analysis revealed a chemically composition based on the compounds of silica, calcium, potassium, aluminum, iron and titanium as well as the presence of groups characteristic to organic compounds. A relatively high number of microbial strains as colony-forming units (CFU) were quantified at $7x10^4$ for the Teişani sample and $1.5x10^5$ for the Malul Alb sample per gram of rock.

Keywords: microbial communities, volcanic tuff, Teişani, Malul Alb, microbial antagonism.

INTRODUCTION

The microbial communities consist of several categories of microorganisms which establish high complexity interactions either between species and metabolic activities. The type of the interaction is highly diverse and could be represented by exclusion microorganisms each other by competition for food, a mutuality crossing also for food but not in the end antagonistic killing. These last types of interaction are in charge for population of microbial community, their dynamic, resilience and stability (Little et al., 2008). Moreover, lots of investigations are highlighting such kind of interactions as important ecological principle for the basis which demonstrates microbial activity in a community (Coyte *et al*., 2015; Garcia-Bayona L and Comstock LE, 2018) and their possible aplication in several industrial, medical or agricultural areas (Lawson CE *et al*., 2019; Tshikantwa TS, *et al*., 2018). One of the most frequently recorded phenomena in an ecosystem appear to be microbial antagonism which confer to microorganisms the ability to design their

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spatial and trophic niches by inhibiting or killing other microbes from their proximity. The phenomena was recorded in 1885 by Victor Babes which noticed that one organism can elaborate a substance which will stop the growth of another (Florey, 1954) giving thus the potential start for developing of antibiotic in medicine (Hutchings MI *et al*., 2019). There a several kind of interaction mechanisms in order to explain microbial antagonism have been analyzed for exploring microbiome engineering. One of them is represented by diffusion based as a consequence of bacteriocins production by microorganisms (Yim and Wang, 2021) and can occur at the level of the cell envelope either by cell wall synthesis inhibition and pores formation or in the cytoplasm through the inhibition of DNA gyrase and RNA polymerase activities (Cotter *et al*., 2013, Pircalabioru *et al.*, 2021).

Between the ecosystems stone appear to harbor a wide spectrum of microbial communities which can colonize the substrate either inside or to outside surfaces (Omelon, 2016) conducting to the development of a biofilm, but the presence of several other microbial communities like heterotrophic, ammonifiers, denitrifiers, sulphatereducing and coliform bacteria were recorded most probably as a consequence of human impact (Batrinescu-Moteau *et al*., 2022a). The rocks consist of minerals ingredients and represent an oligotrophic ecosystem considering the poverty of microbial life which has the ability to colonize it as a consequence of the surface properties like physico-chemical features (Guillitte, 1995; Warscheid and Braams 2000). The microorganisms which line either on the rocks surface or inside represent an important interface between geology and biology and it is important to know how they interact to each other. The rocks colonized by microorganisms could be hard granite or porous like limerstone (Archer *et al*., 2017; Gross *et al*., 1998; Wierzchos *et al*., 2011). As poliextremophilic ecosystems, the endolithic habitats have a diversity of microorganisms relatively less know as metabolic potential and interaction with biologically or ecologically factor their potential (Sajjad *et al*., 2022).

Considering the importance of endolithic microorganisms for their role in biogeochemical cycles, geological and biological records, the aim of this paper was to reveal the antagonist interaction between some bacteria isolated from a volcanic tuff rock due to extracellular synthesis of some factors with such properties, most probably proteins with antibiotic activity.

MATERIALS AND METHODS

Sampling collection and microbial isolation

The fragments of volcanic tuff rocks were taken from areas located near the village Teisani in Prahova county in the vicinity of salt deposit from Slănic Prahova (Figure 1) and Malul Alb in Buzau county close to the salted plateau Meledic. They consist of small fragments kept in appropriate conditions during transfer to the laboratory.

Figure 1. General overview of the sampling site of volcanic tuff rocks in Teisani (upper) and Malul Alb (bottom)

For isolation of bacteria, one gram of milled rock sample was suspended in 10 mL of physiological serum and the resulted solution was used for decimal dilution preparation. The culture medium was represented by MH with following composition (g/L): glucose (1), proteose-peptone (5), yeast extract (10), NaCl (100), $MgCl_2 \cdot 6H_2O$ (7), $MgSO_4 \cdot 7H_2O$ (9.6), $CaCl_2 \cdot 2H_2O$ (0.36), KCl (2), $NaHCO_3$ (0.06), NaBr (0.026) and agar (20) in 1000 mL distilled water (Ventosa *et al*., 1989; Ruginescu *et al*., 2018). The pH of growth medium was adjusted to 7.2 before autoclaving for 30 min at 120˚C. The inoculated plates were incubated at 30^0C for 48 hours and the bacterial strains were purified by streak plate method and obtained purified microbial strains were preserved at 4^0C for furthere experiments.

Fourier Transform Infrared Spectroscopy (FTIR) were used in order to establish main bonds in the volcanic rocks. FTIR spectra were recorded using a Nicolet 6700 spectrometer, at a resolution of 4 cm^{-1}, over the frequency range from 4000 to 400 cm^{-1}. The spectra were taken from transparent KBr pellets. The pellets were prepared by compacting and vacuum-pressing an intimate mixture obtained by grinding 1 mg of rock in 200 mg KBr**.**

The chemical composition and other elements presence in the samples have been determined by XRF analysis using XRF Rigaku ZSX100e, Supermini model (Catana *et al*., 2023; Neagu *et al*., 2021).

Antagonistic activity evidence

In order to perform this test each strain has been tested both target and producer as previously described (Batrinescu-Moteau, *et al*., 2022b; Enache *et al*., 1999; 2004). Thus, target strains were grown in liquid culture medium until they reached stationary phase and then 1 mL of medium containing 10^8 C.F.U./mL has been streaked on plate containing solidified medium. The wells were made in this plate and filled with 50 µL producer strains at equivalent content of C.F.U. After 48 hours incubation the presence of halo surrounding the well was seen as a positive result.

RESULTS

The number of microbial strains as colony-forming units (CFU) isolated from rocks samples was quantified at $7x10^4$ for the Teişani sample and $1.5x10^5$ for the Malul Alb sample per gram of rock as previously presented (Batrinescu-Moteau *et al*., 2022a). As mentioned in a previously paper, the number of CFU is relatively high comparatively with the number characteristic for lakes and other similar environments or rock salt (Cojoc *et al*, 2009). For example, some content in the rocks and the fluids were reported at 5.2×10^3 to 2.4×10^4 cells/g and 3.5×10^8 to 4.2×10^9 cells/g, respectively, counted by methods like acridine orange direct counting and phospholipid fatty acid analysis (Zhang *et al*., 2005). On the other hand a microbial abundance of $\leq10^4$ cells cm^{-3} correlated with the presence of veins in rocks within some depth ranges were also reported (Wee *et al*., 2021). Considering the high numbers of CFU a reach diversity of biologically active compounds secreted in extracellular environment should be expected. The aspect has been observed by the presence of extracellular enzymes, factors with antagonic and antimicrobial activities, exopolysaccharides and other biomolecules as reported in a previously papers (Orhan-Yanikan *et al*, 2020).

The investigated volcanic rocks are natural zeolites with main composition obtained by XRF measurements of aluminosilicates of calcium and potassium. The majority oxide representing between 70% and 74% of the mass of the volcanic rocks is SiO_2 followed by Al_2O_3 about 10 wt % and CaO with 5-9 wt%. Other oxides present in the composition below 5 wt% are: K_2O (4-5%); Fe_2O_3 (3-4.5%); and TiO_2 (1-1.6%). XRF measurements also identified traces (below 1%) of P_2O_5, MnO, NiO, SrO, ZrO_2, SO_3, etc. The volcanic rocks were coloured in light shades, due to finely dispersed impurities such as iron and manganese. The XRF measurements of Teisani rock samples identified the same oxides but in different compositions: SiO_2 37%; Al_2O_3 8-9 %; CaO 12-13 %; K_2O 5-7%; Fe_2O_3 19-20.5% and TiO_2 1-3%. On the other hand the XRF measurements also identified traces of P_2O_5, MnO, MgO, SO_3, etc. The data recorded in the Figure 2 present the FTIR spectra of the volcanic rocks. The interpretation of FTIR spectra was done taking into account the results obtained at XRF measurements. Due to their formation volcanic rocks have amorphous state with small crystallites inside. In all the spectra the highest bands are present at 470 cm^{-1}, 1049 cm^{-1}, 1635 cm^{-1} and near 3444 cm^{-1}. Near main bands are small bands over all domain. The main broad band in the 800–1400 cm^{-1} range with maxima at 1049 cm^{-1} assigned to Si-O-Si stretching vibrations, three or four bands in the 600–750 cm^{-1} range assigned to the presence of Si-O vibrations in the chain of tetrahedral units, a broad band at 470 cm^{-1} associated to the bending motion of the bonds in Si-O evidenced the majoritarian oxide SiO_2 in the volcanic rock composition (Mocioiu *et al*., 2022; Zaharescu and Mocioiu 2013; Zaharescu *et al*., 2008; Mocioiu *et al*., 2017). The bands at 710 cm^{-1} and 790 cm^{-1} are assigned to stretching vibrations of Al_2O_3 (Zaharescu and Mocioiu 2013). The vibrational bands at 671 cm^{-1} and 710 cm^{-1} attributed to bending and asymmetric vibrations of P-O-P (Zaharescu and Mocioiu 2013; Muşat *et al*., 2021). The latter band was attributed to the vibrations of symmetric stretching of P-O-P bridge and overlapped with the vibrations of AlO_4 structural units (Zaharescu and Mocioiu 2013; Muşat *et al*., 2021). The bands at 2505 cm^{-1} and near 3444 cm^{-1} are assigned to water absorbed (Mocioiu et al., 2022; Zaharescu and Mocioiu 2013; Zaharescu *et al*., 2008: Mocioiu et al., 2017). Other small bonds are characteristic to organic compounds from microorganisms bonded by rock. The bands from 2853 cm^{-1} and 2925 cm^{-1} can be assigned to C–H absorptions including CH_2 and CH_3-based compounds (Zaharescu and Mocioiu 2013; Zaharescu *et al*., 2008; Muşat *et al*., 2021; Mocioiu *et al*., 2017). These groups CH_2 and CH_3 are part of almost all organic compounds such as peptide, glucose, proteins, etc (Zaharescu and Mocioiu, 2013; Muşat *et al*., 2021). The bands at 1418 cm^{-1} and 1635 cm^{-1} are assigned to symmetric and asymmetric vibration of the C=O from organic acids (Zaharescu and Mocioiu, 2013; Mocioiu *et al*., 2017). Other inorganic bands are situated below 1000 cm^{-1}. The intensity of bands characteristic of Teisani rock are aparently slow different in agreement with XRF measurements that reported 37% SiO_2 in composition, but the sharp shape of bands characteristic to Si-O bonds show a higher crystallization. Organic bands were observed at 3832 cm^{-1}, 2925 cm^{-1}, 1635 cm^{-1}, 1436 cm^{-1}, in the FTIR spectra and they indicate the growth of microorganisms on the rocks.

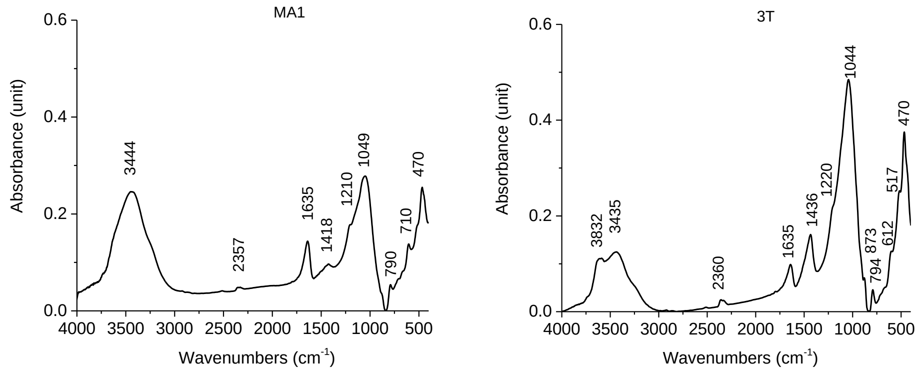

Figure 2. FTIR spectra for the sample of Malul Alb (left) and Teisani (right)

Between investigated strains from *Teisani rock* 40 acting as antagonistic factors (AF) producers have the ability to kill other 19 strains (Figure 3, 7). According to their spectrum of action the producers of AF can be grouped in three categories. The first one (A category) consist of 33 strains which act towards one to five strains (Figure 4), the second one (B category) from three strains (48, 49 and 51) which kill between six and ten strains and last one (C category) with four strains (16, 42, 43 and 45) acting on more than 11 strains (Figure 5). When the strains are analyzed as target of AF they can be grouped in four categories noted as S (sensitivity to AF between 1 and 5 strains), S1 (sensitivity from 6 to 10 strains), S2 (between 11 and 15) and S3 (sensitivity to over 16 strains). In the S categories are included six strains (10, 18, 19, 21, 22 and 23), in S1 eight (2, 3, 4, 5, 6, 8, 9 and 12), in S2 three strains (1, 15 and 54) and in the last category S3 two strains, 7 and 14 respectively (Figures 4 and 5). On the other hand, from a total, of 40 investigated strains 21 were no inhibited by AF producing strains, but nine strains (1, 6, 10, 12, 14, 15, 19, 21 and 54) acted both as producers and target of AF. As producers of AF they are included in A category and as target in S (strain 10), S1 (strains 6 and 12), S2 (strains 1, 15 and 54) and in S3 (strain 14) categories respectively.

The evaluation of the potential antagonistic activity of the isolated strains from *Malul Alb rock* (Figures 6, 7) revealed the fact that from a total of 24 isolates four were not inhibited by those producing factors with antagonistic activity. On the other hand, with the exception of five isolates (1-4, 3-6, 3-9, 3-10, 4-3) the remains have capacity to produce AF. On the other hand, the isolates 1-6, 4-1 and 4-5 stand out as FA producers, and isolates 1-1, 1-4, 1-6 and 4-6 stand out as targets for antagonic factors. Based on their acivity spectrum the AF producing isolates can be grouped into two categories, namely with action on a number of 1-3 isolates (11 isolates) or 4-7 isolates (8 isolates). These results indicated strains 1-2, 1-5, 1-7, 3-2, 3-5 and 4-4 as a promising candidate for test for potential antimicrobial activity against pathogenic strains. Following the obtained results the strains MS1-2 and MS1-7 show ability to kill *Staphyloccous aureus* and strain 1-7 acting also against *Lysteria monocitogenes*. The recorded data confirm no activity for all strains against *Escherichia coli*, *Salmonela typhyrium* and *Pseudomonas aeruginosa*. These preliminary data encouraged further approach for positive results in order to identify the extracellular biologically active products as alternative for classical antibiotics treatments. On the other hand the increasing demand for fresh fruits and vegetables need novel strategies for their biopreservation during transportation and handling considering the decayed of these products by pathogens during postharvest (El-Ghaouth et al., 2004; Droby, 2006; Zhu, 2006; Singh and Sharma, 2007). Environmental pollution due to chemicals use conducted to the necesity of antagonic microbial producers to be used as biologically alternative in this way (Sharma et al., 2009). These biomolecules can be used as biocontrol agents and the sources from microorganisms isolated from environments with extreme or hostile conditions like these which consist of the subject of this study can offer an economically viable alternative.

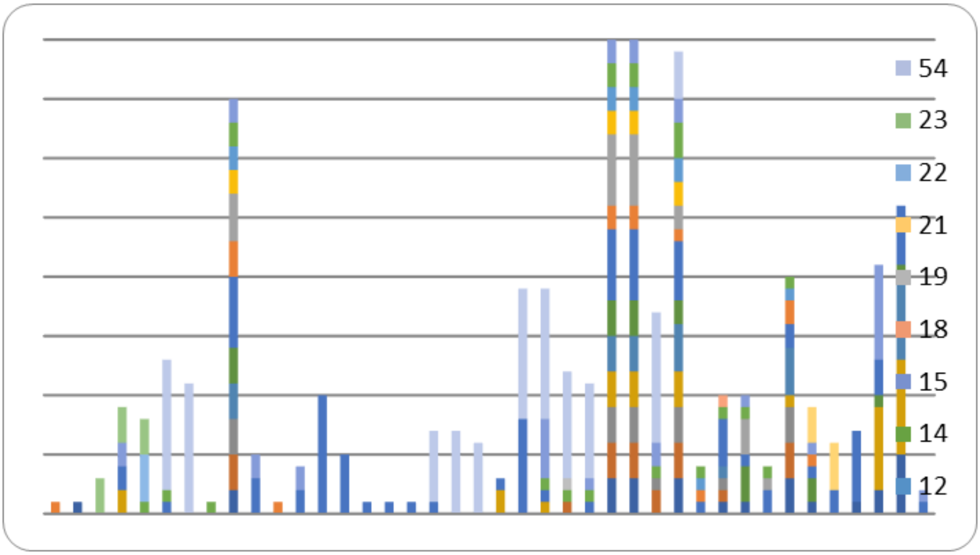

Figure 3. The production of factors with antagonistic activity (AF) from the microbial strains isolated from Teisani rock. The number from the horizontal ax indicated the AF producing strains.

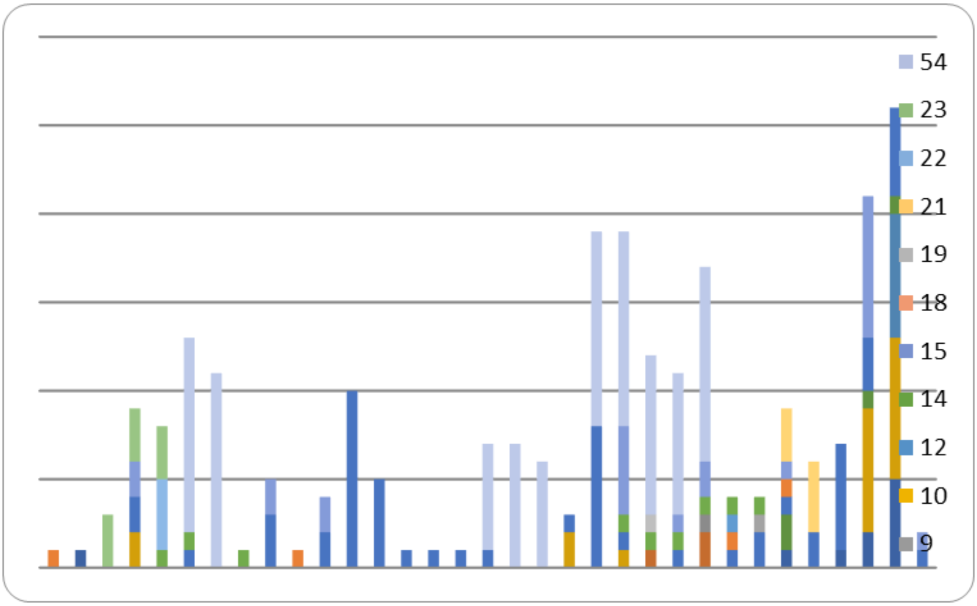

Figure 4. The AF producing strains isolated from Teisani rock and grouped in first category (A) of activity spectrum. The number from the horizontal ax indicated the AF producing strains.

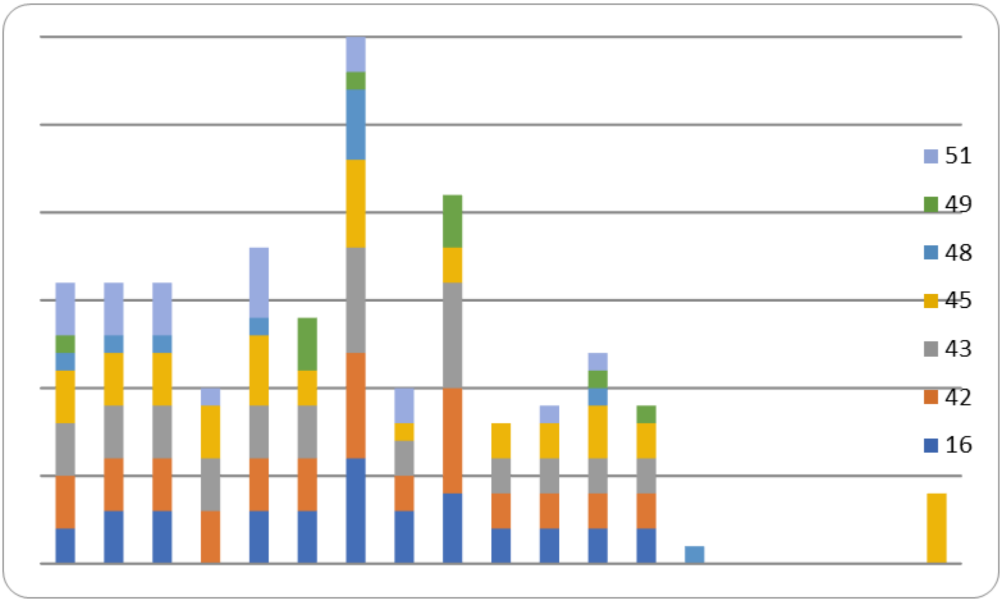

Figure 5. The AF producing strains isolated from Teisani rock and grouped in the second and the last categories (B and C) of activity spectrum. The number from the horizontal ax indicated the AF producing strains.

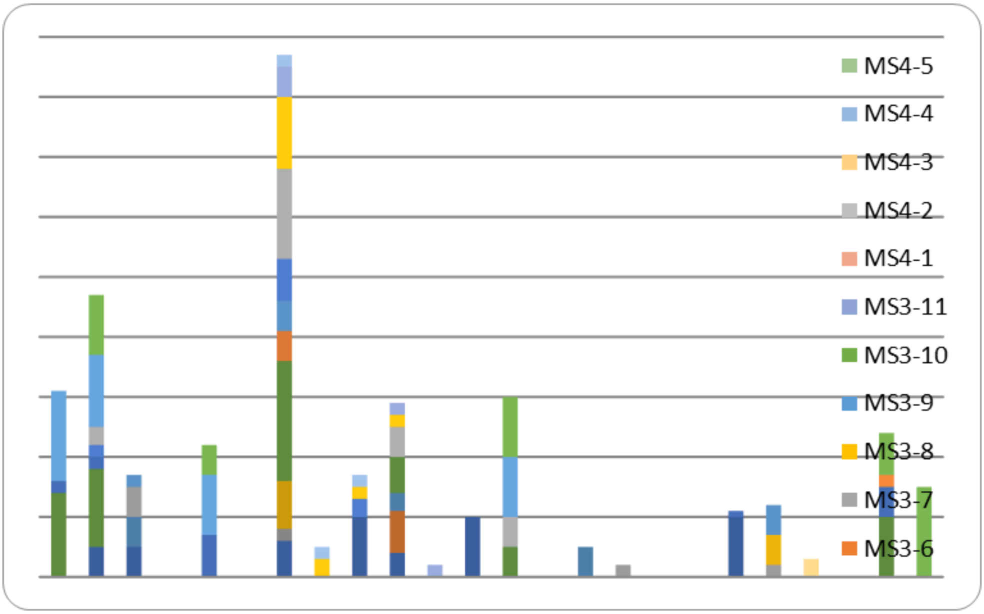

Figure 6. The production of factors with antagonistic activity (AF) from the microbial strains isolated from Malul Alb rock. The number from the horizontal ax indicated the AF producing strains.

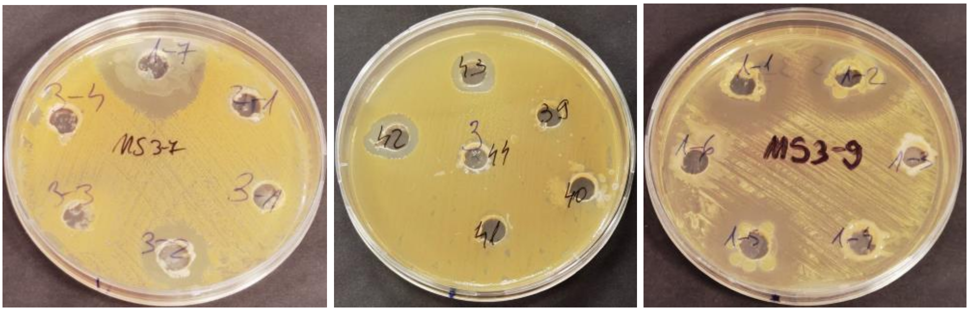

Figure 7. Evidence of AF producing by several investigated strains from Malul Alb (right and left) and Teisani (middle).

CONCLUSIONS

The results obtained in this study revealed the capacity of some investigated strains to synthesis some factors with antagonistic activity (AF) which can kill other strains isolated from the same environments or some pathogenic strains. Between 64 tested strains from two types of volcanic tuff rock 59 have the ability to synthetize AF. Some of the strains (1-2 and 1-7 respectively) are acting against pathogenic strains *Staphyloccocus aureus* and *Lysteria monocytogenes*. These data give the opportunity for further studies to describe the AF obtained from microbial cultures as alternative to classical chemicals therapeutic antibiotics (Cotter *et al*., 2013; Allen *et al*., 2014). On the other hand, the biomolecules like AF were proposed to be used in others industrial area with major economic impact like biocontrol agents (Ren *et al*., 2020), due to their inhibitory behaviors against some bacteria and fungi. As mechanisms of action towards target microbial strains the AF from this study are oriented for physically competing for nutrients and living space (Palumbo *et al*., 2006) considering also the chemically composition and rock structure from which the bacterial strains producing them were isolated. Thus, the functional characteristics of studied AF give a glimpse on unraveling the dynamics of the microbial community associated with the volcanic tuff rock for further describe the natural biogeochemical cycles and biologically mechanisms for microbial community development in such type of extreme environments.

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