Effect of the Type of *Vitis vinifera* Cultivation in the *Cenophenoresistome* and Metabolic Profiling (CLPP) of Edaphic Bacterial Communities

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Abstract: In the present work, bacterial soil communities of different grapevine exploitation samples are studied in order to elucidate the possible influence of different agrarian management techniques (use of fertilizers, irrigation with river water) may have on the rhizospheric microbiome of *Vitis vinifera* plants. Therefore, it is postulated the *Cenophenoresistome* as a novel methodology to evaluate complex communities' global resistance against different antibiotics, by using and adjusting a serial of techniques traditionally applied to evaluate a monospecific population's resistance against antibiotics (Vitek®, ATB® and disk diffusion methods). Likewise, the metabolic profile (CLPP: community level physiological profile) of bacterial communities is studied by Biolog ECO®. In relation to the functional structure of the bacterial communities, it is observed that the metabolic profile (diversity, kinetics and CLPP) of unexploited soils differs from soils under anthropic influence. It is discussed the causes of resistance in the human clinic antibiotic treatment based on the agrarian management, especially with the contamination transmitted by irrigation water, which could be associated with changes in edaphic communities. The results obtained in the present study through two different approaches (*Cenophenoresistome* and metabolic profiles) are consistent with each other, suggesting that both methods can be good bioindicators of the state of humankind-altered soils that host natural ecosystems. Likewise, the concept of *Cenophenoresistome* is proposed as a bioindicator of soil response to alteration processes, as well as a possible predictor of its evolution in edaphic remediation processes.

Key words: Bacterial communities, antibiotic resistance, *Cenophenoresistome*, *Vitis vinifera*, community level physiological profile, Biolog ECO.

1. Introduction

Spain is one of the world's largest wine producers [1]. *Vitis vinifera* adapts to a wide variety of terrains. At present, it is estimated that there are 8,000 varieties of grapevines, including wild varieties. New vines are still recently described, and others have been lost over the years.

Soil is the terrestrial ecosystem that presents the largest species abundance [2], being the most abundant and diverse group, the one constituted by bacteria. Soil microorganisms play an important role in maintaining soil quality and can be sensitive to

environmental disturbances [3]. Microbial diversity has therefore been proposed as an indicator of soil quality [4], as far as soil microbial diversity is related to its ability to cope with potential disturbances [5]. Thus, the analysis of the diversity of edaphic microbiota (whether metabolic or structural) is essential when studying environmental and anthropogenic influences on soil quality [6].

On the other hand, soil microbiota represents one of the earliest evolutionary origins of antibiotic resistance, and has been proposed as important reservoir of resistance genes [7] that may be horizontally transferred between soil bacteria and pathogenic bacteria, as these microorganisms, even in natural environments free of antibiotics, can carry a

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large number of resistance genes [8]. This resistance to antibiotics is a natural biological evolutionary phenomenon that can be accelerated by various factors, especially by human practices [9].

The soil is not only in direct contact with antibiotics due to livestock and agricultural activity, but also the natural habitat of numerous natural antibiotic producing species, such as *Streptomyces* spp. Most intrinsically resistant bacteria have a non-clinical origin, which have no antibiotic selective pressure equivalent to that which can be given in a hospital setting [10].

Some water resources have become reservoirs of antibiotic resistant genes that can be transferred to pathogens in aquatic environments and, eventually, cation in the soil, associated with the root of the plants [11]. Many authors indicate that the discharge of treatment plants contributes to the propagation of antibiotic resistance genes by the environment and causes an impact on the bacterial communities of the receiving river [12, 13] and finally, to the human clinic [14].

Currently, antimicrobial resistance poses a serious threat to public health and requires a better understanding of the genes responsible for such resistance [15], its selection and its propagation through the environment [16]. This problem is not limited to the hospital environment, but the use and even the abuse of antibiotics in animal production and agriculture have also led to an increase in the prevalence of antibiotic resistant microorganisms in different ecological niches. Thus, in order to minimize the different impacts on human health, there is a scientific interest in finding solutions to address the environmental contamination, as well as indicators that allow the evaluation of soil biological alterations. Therefore, microbial soil diversity is related to its ability to cope with potential disturbances [17], and the phenotypic profile of antimicrobial resistance is considered an important indicator of microbial activity [18].

The objective of this work was to study the differences in soil bacterial communities, attending to the distinct agricultural uses in the exploitation of *V. vinifera* plantations. In order to know the microbiological quality of the irrigation water, the microorganisms present in the Tajuña river water have also been studied to further understand its potential influence in the soil bacterial communities.

2. Materials and Methods

2.1 Sample Collection

Vine soil samples were collected in the town of Tielmes, municipality of the Community of Madrid (Spain), belonging to the region of Las Vegas (latitude: 40.2358716, longitude: -3.3007076). Its soils are slightly alkaline and are composed mainly of marls and gypsum from the tertiary period. It is traditionally an agricultural region. The soils of the area are considered of great fertility, and are under the influence of the Tajuña river.

Soils in agricultural exploitation are subject to different uses. The following eight samples were taken: soil with no agricultural use (A), abandoned vineyard crop (B), vine on holding fertilizer (C), vine on farm (D), vine on olive grid (E), vine on fertilized olive tree grid (F), vine in almond tree grid (G), vine in fertilized almond tree grid (H). Samples C, D, E, F, G and H correspond to soils in operation irrigated with water from the Rio Tajuña. Samples A and B are used as a control of the possible influence that the different uses mentioned above can have, since they have a different historical use and they are currently neither irrigated nor fertilized.

All soil samples were collected in sterile plastic bags and transported in a refrigerator (4 °C) and processed. On the same day, a sample of water from the Tajuña was collected and stored in the same conditions previously mentioned.

2.2 Extraction of Edaphic Bacterial Communities

Once in the laboratory, the extraction of the

microorganisms was performed based on a modified procedure described by García-Villaraco et al. [19]. Briefly, 2.5 g of each sample were weighed, carefully selected to avoid useless material in the sample. Each sample was suspended in sterile 0.45% saline solution to a final volume of 25 mL and homogenized to ensure complete extraction of microorganisms. This last step consists of a first step of 16,000 rpm for 2 min in a minifugue, an intermediate step stirring at 2,800 rpm for another 2 min and finally 10 min in the centrifuge at 2,500 rpm. The resulting supernatant constitutes the sample dilution 10^{-1} with which the different tests are performed. The water sample was analyzed directly without dilution of the sample.

2.3 Bacterial Count

A semi-quantitative count in laminoculture (Uritest®) was performed according to the manufacturer's specifications. The 10^{-1} dilutions were directly plated and incubated at 37 °C for 24 h for subsequent counting. In the case of water, a laminoculture was also made by directly sowing by immersion.

The results shown in the present work were obtained by performing the arithmetic mean of three replicates.

2.4 Adequacy of Methods to Obtain the *Cenophenoresistome*

Since there is no previous reference in the literature to the *Cenophenoresistome* concept, in order to obtain its value, it was necessary to adapt methods traditionally used to obtain the profile of antibiotic sensitivity of isolated microorganisms. In all cases, a standardized 0.5 McFarland inoculum was used, which corresponded to the 10^{-1} dilution from the soil sample extraction.

2.5 Modification of the Disk Diffusion Method

The Kirby-Bauer method was used under EUCAST recommendations [20]. The method includes the use

of Mueller-Hinton agar without supplements. The modification of the method consisted in the use of the inoculum described above. The following antibiotics were tested: ampicillin (25 µg), ceftazidime (30 µg), cefepime (30 µg), gentamicin (25 µg), nalidixic acid (30 µg) and co-trimoxazole (30 µg) (sulfomethoxazole with trimethoprim). They were incubated at 37 °C for 24 h. After a confluent growth, the antibiogram was read for the interpretation of the zone diameters in combinations of species in individual agents and the zone diameter cut-off points were calibrated for the minimum inhibitory concentration (MIC). Given the dominance of Enterobacteriaceae in this type of soil, the antibiograms were interpreted according to the rules of the EUCAST committee for this microbial group.

2.6 Modification of BioMerieux® ATB G-EU (8)® Galleries

Exploratory tests were carried out. The modification of the method was proposed after the inoculation of the gallery with volumes of 10, 50 and 100 µL of the inoculum described above in 7 mL of ATB medium. In all cases, after homogenization, 135 µL were taken and added to each well of the gallery, and incubated for 24 h at 37 °C. Finally, the optical density was read. By comparison with the reference method [20], it was observed that the results that showed the greatest similarity were obtained by adding 100 µL of inoculum to the 7 mL of ATB medium. In the present work, only the results obtained under these conditions are included.

2.7 Modification of Vitek® BioMerieux®

Exploratory tests were performed in order to find the adequacy that more accurately reflects the *Cenophenoresistome*. For this, different inoculum volumes of 1, 2 and 3 mL were tested. Inoculation was carried out in the gallery Vitek2™ N-243. Incubation and reading were performed under the conditions described by the manufacturer. By

comparison with the reference method [20], it was observed that the results that showed the greatest similarity were obtained when 3 mL of inocula were added. In the present work, only the results obtained under these conditions are included.

2.8 *Cenophenoresistome* Calculation of the Different Communities

Inoculation was carried out in the gallery Vitek2™ N-243 with a 3 mL inoculum. Incubation and reading were performed under the conditions described by the manufacturer.

2.9 Metabolic Profiles of Bacterial Communities

The community level physiological profile (CLPP) was performed using Biolog ECO® [21]. The plates used in this study (Biolog EcoPlates®, Biolog Inc., Hayward, CA, USA) consist of 96 wells containing different sources of lyophilized carbon and nitrogen nutrients. Wells of the Biolog ECO® plates were loaded with 135 µL using a multichannel pipette from the 10⁻¹ microbial suspension. This process was repeated for each of the soil. The plates were incubated at 27 °C and the absorbance was measured at 590 nm, at 61, 85, 109, 135 and 159 h of incubation.

With the absorbance data, for each treatment and measurement time, each value was corrected by subtracting the blank (corrected absorbance). Corrected mean absorbance of all the wells was calculated, as the mean of the 31 absorbance values was adjusted for each replicate.

Next, the average well color development (AWCD) value *versus* the incubation time was plotted to obtain the growth curves of the microbial community in the wells of the plate (community kinetics). In these curves, the incubation moment was chosen when the growth of the microorganisms was initiating the stationary phase, in our case, 135 h. With the corrected absorbance values for the time of incubation chosen, the metabolic diversity of each sample was calculated by using the Shannon-Weaver diversity

index (*H*):

$$H(m) = -\sum q_i \log_2 q_i \quad (1)$$

where, $q_i = n/N$, n is the corrected absorbance (AWDC) at 590 nm of each well of Biolog ECO®, and N is the total absorbance of all wells.

2.10 Data Analysis Treatment

The analysis of the pre-processed data was done by principal component analysis (PCA) using the SPSS v. 19.0 statistical program (SSPS Inc.). These multivariate analyses were carried out both to evaluate the similarities between the different samples and to discriminate the loading factors of that order (carbon sources of the Biolog ECO® plate).

3. Results and Discussion

3.1 Bacterial Count

The number of bacteria in different soils was analyzed to see if they were comparable, in order to avoid bias due to variations in total amount of cultivable bacteria. Since a similar order of magnitude was obtained, it was assumed that the microorganism communities were comparable both to assess the *Cenophenoresistome* and to compare CLPP. A greater number in these populations could lead to false positives of antibiotic resistance, interpreted erroneously as an increase of resistance as an effect of anthropic activity. The results of the semi-quantitative count in laminoculture (Uritest®) in both water and soil showed that in all samples, the MC/*Escherichia coli* density was 10³ cfu, while in the case of CLED, the amount was 10⁴ cfu in A, B and G, and 10⁵ cfu for the other samples. In the case of water, the CLED was 10³ cfu.

3.2 Comparison of the Methods for Obtaining the *Cenophenoresistome*

Kirby-Bauer diffusion disc method results were used as reference. Results obtained using automatic and semi-automatic techniques were compared under the conditions described above. In all cases, the

EUCAST v. 7.0 (2017) interpretation criteria [22] were used. Despite being a reference technique widely used for the characterization of antibiotic resistance in a population, it was found a case series of results difficult to interpret when used to test the behavior of a community. Fig. 1 showed the different types of halos.

For the subsequent statistical analysis, the anomalous results were not considered so that it was necessary to repeat each test until three replicates could be correctly evaluated. In cases where more than one halo appeared, the most restrictive was valid as long as it was well defined.

In order to obtain the resistance profile of a community, automatic and semi-automatic techniques may show false positives due to the growth of fungi in reading areas of the well. This does not happen in the evaluation of the disc diffusion method, since in this technique, microbial growth can directly be observed.

However, these techniques may have a clear advantage over traditional methods in interpreting the result in that they yield an objective numerical data of

MIC, from which the behavior of the population is inferred according to the criteria of the EUCAST committee. Thus, erroneous interpretations are avoided in the heterogeneous manifestation of inhibition halos when tested for polymicrobial communities. In the same way, the automated techniques are more comfortable allowing the community to test against numerous antibiotics in the same gallery.

Next, as shown in Table 1, the total number of resistances, sensitivities or intermediates of the disk diffusion method was compared with respect to the ATB[®] technique and with respect to Vitek[®] obtaining the percent similarity index as set in Table 2. As shown in Table 2, Vitek[®] presented a higher mean homology percentage to the reference disk diffusion method (91.67%), in comparison with the 64.58% of homology of the ATB[®] method with the same reference pattern. As the similarity index collects higher results using the Vitek[®], this technique will be used to obtain the *Cenophenoresistome*, under the EUCAST criterion for Enterobacteriaceae. The abundance of this group of microorganisms coincides

Fig. 1 Photographs of the different types of inhibition halos in four examples of the disk diffusion technique.

with previous studies that postulate the importance of these in edaphic ecosystems [23].

3.3 *Cenophenoresistome* Analysis

The resistance effect in the different populations may be due to different factors, including the additive effect by different strains mediated by microbial communication processes, such as quorum-sensing/quorum-quenching, as well as the response to abiotic factors. Therefore, the explanation regarding the entire resistance profile of the communities against a particular antibiotic will be due to different mechanisms of resistance expressed by the different strains that compose them. The complete results of the *Cenophenoresistome* were shown in Table 3. These mechanisms of interaction between strains could lead to antibiotypes impossible for a particular population. However, analyzing the results shown in Table 4, it's seen that communities have consistent responses in their phenotype against different groups of antibiotics. Following the same criterion of resistance consistency against the different families of antibiotics, it was found that the results of the *Cenophenoresistome* are comparable to each other

and allow the comparison of the impact that different agricultural uses have on the same type of soil.

Also, considering the results obtained and the approaches offered by Vitek®, it can be inferred that in these communities, the following mechanisms of resistance could be found.

Analyzing the results of the β -lactams group, it's found in all cases that resistance to penicillins is very abundant. This is not the case when they are accompanied by β -lactamase inhibitors, which is completely consistent.

Gram negative bacteria are very abundant in the rhizosphere of plants. One of the most abundant groups of these bacteria in the rhizosphere (SR) belongs to the *Pseudomonas* genus [24]. These microorganisms present intrinsic resistance to several groups of antimicrobials [25]. This natural resistance is associated, in some of its species, with a low permeability of its outer membrane partly related to the functional capacity of the main porin. For example, the OprF of *Pseudomonas aeruginosa* significantly limits the passage of antimicrobials. In recent years, different active efflux systems (efflux pumps) have been described. These systems confer pleiotropic

Table 1 Results of *Cenophenoresistome* of different samples and treatments interpreted according to EUCAST v. 7.0 (2017) criteria.

	A			B			C			D			E			F			G			H		
	Kirby-Bauer	ATB®	Vitek®	Kirby-Bauer	ATB®	Vitek®	Kirby-Bauer	ATB®	Vitek®	Kirby-Bauer	ATB®	Vitek®	Kirby-Bauer	ATB®	Vitek®	Kirby-Bauer	ATB®	Vitek®	Kirby-Bauer	ATB®	Vitek®	Kirby-Bauer	ATB®	Vitek®
AMP	I	I	I	I	S	I	I	R	R	I	R	R	I	R	R	I	S	R	R	S	R	I	S	R
NA	R	R	I	R	S	R	R	R	R	S	R	R	I	R	R	R	R	R	R	R	R	R	R	R
CAZ	I	R	I	R	R	I	S	S	R	R	S	R	S	S	R	R	R	R	I	S	R	R	R	R
FEP	S	R	S	R	S	I	I	R	R	I	I	R	I	I	R	R	I	R	S	R	R	I	R	R
GN	S	S	S	S	S	S	R	S	I	I	S	I	S	S	S	S	S	S	S	S	S	S	S	S
SXT	S	S	S	S	S	S	I	S	R	S	S	S	S	S	S	I	S	I	I	S	I	S	S	S

A: soil without exploitation; B: abandoned vine soil; C: fertilized vine soil; D: vine soil in operation; E: vine soil in olive grid; F: vine soil in olive grid fertilized; G: vine soil in almond grid; H: vine soil in almond grid fertilized.

AMP: ampicillin; NA: nalidixic acid; CAZ: ceftazidime; FEP: cefepime; GN: gentamicin; SXT: cotrimoxazole.

S: sensitive; R: resistant; I: intermediate resistance.

It is highlighted in grey shading the coincidences in the three methods under the criterion that I and R correspond to antibiotic resistance.

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Table 2 Similarity index (%) of the Vitek® and ATB® methods with respect to disk diffusion.

	A (%)	B (%)	C (%)	D (%)	E (%)	F (%)	G (%)	H (%)	Total (%)
ATB®	83.33	50	66.66	50	83.33	66.66	33.33	83.33	64.58
Vitek®	100	100	83.33	83.33	83.33	100	83.33	100	91.67

A: soil without exploitation; B: abandoned vine soil; C: fertilized vine soil; D: vine soil in operation; E: vine soil in olive grid; F: vine soil in olive grid fertilized; G: vine soil in almond grid; H: vine soil in almond grid fertilized.

Table 3 *Cenophenoresistome* of the community obtained using Vitek® under the described conditions.

Antibiotics	Use							
	A	B	C	D	E	F	G	H
Ampicillin	I	I	R	R	R	R	R	R
Amoxicillin/ac. clavulanic	S	S	S	S	S	S	R	S
Piperacillin/tazobactam	S	S	S	S	S	S	S	S
Cefuroxime	I	I	R	R	R	R	R	R
Cefuroxime-axetil	I	I	R	R	R	R	R	R
Cefoxitin	S	S	R	R	R	R	R	R
Cefotaxime	I	I	R	R	R	R	R	R
Ceftazidime	I	I	R	R	R	R	R	R
Cefepima	S	I	R	R	R	R	R	R
Ertapenem	S	S	R	I	R	I	I	R
Imipenem	S	S	I	S	S	S	S	S
Amikacin	S	S	I	S	S	S	S	S
Gentamicin	S	S	I	I	S	S	S	S
Nalidixicacid	I	R	R	R	R	R	R	R
Ciprofloxacin	S	S	R	S	S	S	S	R
Tigecycline	S	S	R	R	S	R	R	R
Trimethoprim/sulfamethoxazole	S	S	R	S	S	I	I	S

A: soil without exploitation; B: abandoned vine soil; C: fertilized vine soil; D: vine soil in operation; E: vine soil in olive grid; F: vine soil in olive grid fertilized; G: vine soil in almond grid; H: vine soil in almond grid fertilized.

S: sensitive; R: resistant; I: intermediate resistance.

It is highlighted in grey shading the results of communities that are sensitive to different antibiotics.

Table 4 Possible resistance mechanisms in the different soil fields.

Possible mechanisms of resistance	Agricultural uses							
	A	B	C	D	E	F	G	H
AmpC	Ps	Ps	H	P	P	H	H	H
BLEE	A	A	P	A	P	P	P	P
Carbapenemases	A	A	Ps	A	A	A	A	Ps
AAC	A	A	P	P	A	A	A	A
QRDR mutation	A	A	P	P	P	P	P	P
TET	A	A	P	P	A	P	P	P
Mutations in enzymes folic acid synthesis pathway	A	A	P	A	A	P	P	A

P = presence; Ps = possible presence; A = absence; H = hyperproduction.

AmpC = β -lactamase type AmpC; BLEE = extended spectrum β -lactamases; AAC = N-acetyltransferase, adenylase and/or phosphatase; TET = tetracycline resistance genes; QRDR mutation: mutations in the quinolone resistance-determining region.

A: soil without exploitation; B: abandoned vine soil; C: fertilized vine soil; D: vine soil in operation; E: vine soil in olive grid; F: vine soil in olive grid fertilized; G: vine soil in almond grid; H: vine soil in almond grid fertilized.

The mechanisms of resistance described in human clinical pathogens and less expected in a soil are highlighted in shading.

resistance to bacteria [24].

The abundance of bacteria from the *Pseudomonas* group in the fraction of the soil that can be cultured could explain this resistance to ampicillin, as well as the resistance of penicillins in combination with inhibitors of β -lactamases (amoxicillin/clavulanic acid). *Pseudomonas* spp. expresses a class C chromosomal β -lactamase (AmpC, cannot be inhibited by the usual β -lactamase inhibitors, such as clavulanic acid), which contributes to resistance to many β -lactams, including penicillins. Other gram negative species, such as *Stenotrophomonas maltophilia*, *Burkholderia* spp., *Achromobacter xylosoxidans* also express intrinsic resistance mechanisms against penicillins [26].

The same happens when analyzing the *Cenophenoresistome* against cefotaxime and ceftazidime (3rd generation cephalosporins). In the authors' analysis, it's found elevated MIC in soils C, D, E, F, G and H. These antibiotic resistances can be explained due to the fact that although it has an inducible character in isolates of the *Pseudomonas* genus, virtually all species of this genus express the chromosomal type AmpC β -lactamase. The expression of this enzyme contributes to the resistance against many of the β -lactams, including penicillins and cephalosporins of the first and second generation, cephamycins and many of the third generation cephalosporins, including cefotaxime and ceftazidime.

Based on the results obtained in the case of irrigated soils with waters from the Tajuña river and in operation (C, D, E, F, G and H), their resistance to cephalosporins and ampicillins can be explained by the presence of strains (β -lactamases of extended spectrum), unlike soils that are not watered by these waters and are found without exploitation (A) or abandoned (B), whose sensitivity to ampicillin is explained by being a community consisting of mostly wild strains with a sensitivity profile. All C, F and H soils share the fact of being organic matter receptors in the form of fertilizers. In all of them, resistance profiles are compatible with the presence of AmpC

hyperproductive strains, which suggests that it could be the result of a notable anthropic effect.

There are no significant differences compared to imipenem. Likewise, in the vast majority of cases, the resistance against imipenem in *P. aeruginosa* depends on the combination of the production of AmpC and the loss of the porin OprD, the production of carbapenemases or alteration in the expression of PBP 4 [27, 28]. These events have been only described in isolated of clinical origin and in a very low incidence. The irrigated soils are resistant to ertapenem while they exhibit sensitivity to imipenem. Soils without any exploitation (A and B) show sensitivity to this type of β -lactams.

Ertapenem is effective against gram negative bacteria, anaerobic bacteria and bacteria with ESBL, but it is not effective against *P. aeruginosa*. This could be the explanation of the levels of resistance that are observed in soils with agricultural use that carry a greater anthropic pressure. This generally happens when comparing soils of different agricultural uses. Higher levels of resistance are found in soils in exploitation (C, D, E, F, G and H) than in soils that harbor natural ecosystems (A) or abandoned crops (B). This responds to the anthropic pressure on the first ones since they are receivers of irrigation from waters with a high contamination of fecal origin. Imipenem has a broad antibacterial spectrum that includes gram negative bacteria, both aerobic and anaerobic. It is especially potent against *P. aeruginosa*. It is stable in the presence of β -lactamases (penicillinase and cephalosporinase) produced by different bacteria. It acts as a potent inhibitor of β -lactamases of gram negative bacteria that is resistant to most β -lactam antibiotics. This explains why these communities, in which it is possible to find abundance of *Pseudomonas* spp., present profiles of greater sensitivity than the one against ertapenem.

The most common acquired resistance to aminoglycosides is due to the enzymatic inactivation of the antibiotic by aminoglycoside-modifying

enzymes (acetylases, adenylase and phosphatases) [29]. The susceptibility to aminoglycosides presented by the communities corresponding to soils A, B, E, F, G and H could be explained by a higher prevalence of wild strains in the community, although in some soils (C and D), an intermediate resistance is due to the presence of strains that could possess AAC as an enzyme with inactivating power.

As for the quinolones, it was found greater resistance against nalidixic acid than against ciprofloxacin. This result is completely consistent since fluoroquinolones improves their spectrum against bacteria [30]. The use of fluoroquinolones has been associated with the development of resistance, not only to this group but to other groups of antibiotics, both individually and in the community. In the clinical setting, the resistance cross-selection processes are well described. Of particular concern is the relationship between the use of fluoroquinolones and the increase of enterobacteria with ESBL. This fact is manifested in the soil samples with uses of C and H, both with vine in operation and recipients of organic fertilizers and irrigation waters with high fecal content. Resistance to quinolones is mainly explained by the presence of point mutations in the QRDR region of the coding genes for gyrases and topoisomerases. The quinolones are antibiotics that do not come from biological synthesis, but are chemically synthesized [30], which could support the idea that the presence of strains expressing these resistances has an origin or selection of anthropic origin. Likewise, the strains that carry these mechanisms retain them long after they have remitted the pressure that gave rise to them so they can attest the contribution of contamination many years after abandoning these practices.

In the case of tetracycline, resistance can be explained as a consequence of the acquisition of bacterial resistance genes [31]. In this study, resistance to tetracyclines was found in soils C, F and H. These three types of soils are fertilized and receive

contaminated water for irrigation. Soils D and G also receive the contribution of these irrigations, but do not get any fertilizer contribution. This could indicate that the contamination that they manifest could be carried through the water.

3.4 Metabolic Profiles of Bacterial Communities

3.4.1 Kinetics of Bacterial Communities

Fig. 2 showed the AWCD value obtained on the Biolog ECO[®] plates, *versus* the incubation time of each sample. This graphical representation gives the idea of the temporal dynamics of bacterial communities submitted to the different management when growing in plates and of its metabolic functionality.

Based on the literature reviews of this technique and the plate counting, the absorbance was measured after 61 h, since it is when differences in the metabolic activity of all samples start to appear.

From there, an increase in absorbance occurs in all samples, resulting in a greater slope in bacterial activity and *vice versa*, until it reaches a maximum point prior to the stationary phase, where it is considered that they have reached their maximum activity. In this case, it is considered that its maximum is 135 h.

It is observed that the soils with less activity are those without direct anthropological effect by labor, fertilization or irrigation, in this case, natural soil (A) followed by abandoned vine (B).

On the contrary, the ones that have shown the greatest activity are those soils that were fertilized and in mixed cultures with other plants. Both the water and the cultivated vine soil (D) have an intermediate activity among the previous ones.

These results showed that the anthropological effect increased the rates of microbial activity. This may be due to increased input of organic matter through fertilization and increased availability and heterogeneity of carbon sources. This effect has already been observed by other authors in other types of crops [32]. However, the effect of irrigation on exudation of plants cannot be ruled out [33], which

Fig. 2 Graphical representation of the AWCD value obtained in the Biolog ECO[®] plates, against the incubation time of each of the studied uses and water.

The average absorbance of the three replicates per sample is shown in the axis.

could increase the metabolic rates of soil bacterial communities. Finally, the addition of microorganisms with irrigation could also imply an increase of kinetics by increasing the number of microorganisms in the soil [34, 35].

3.4.2 Metabolic Diversity

Fig. 3 showed the metabolic diversity values found in each sample studied. It can be observed a relative connection with microbial kinetics, since the soils without exploitation (free and abandoned) have less diversity, from which, the greater diversity is found in the abandoned, which has had some use in the past. This soil has diversity similar to that of water, which is followed by the vine soil in exploitation (D) being those with a higher value of mixed crops.

These results contrast with those reported by Carrera et al. [36], in which the addition of organic

matter with increased metabolic diversity is related. In this case, this relationship is not found. The use of agrochemicals does not diminish diversity as indicated by Johnsen et al. [37], on the contrary, less diversity in those treatments in which agrochemicals were not used (non-exploited soils: A and B) has been found in this work.

3.4.3 Differences in the CLPP of the Communities

Analysis of main components (ACP) was performed with the overall results for all replicates and with the 31 carbon sources of Biolog ECO[®] for each of the samples. The first two components absorb 68.097% of the variance, explaining the first one 50.51% and the second one 17.58%.

Fig. 4 showed the ACP representation of the Biolog ECO[®] samples (CLPP in the different samples) projected in the first two components.

Fig. 3 Metabolic diversity ($H(m)$) values obtained from the absorbance values at 135 h on the Biolog ECO® plates. Average of three replicates for each sample and the standard error bars are plotted. Units of ordinates are represented in bits.

Fig. 4 ACP representation of the Biolog ECO® samples (CLPP in the different samples) projected in the first two components.

★ = fertilizer crops; ➡ = mixed crops.

Fig. 5 Representation of the ACP loading factors of the Biolog ECO® samples (CLPP in the different samples).

There are two large, well-differentiated groups that show different analogies regarding the use of carbon and nitrogen sources.

In one side, there are unused soils (A and B), on the other one the remaining soils (C, D, E, F, G and H) and the water. The common factor that presents this last group is the irrigation with effluent waters. In turn, it is seen that this second large group has greater similarity between subscribed soils (C, F and H).

Finally, it does not seem that there is a clear relevance in the metabolic profile if the culture is or is not mixed.

Fig. 5 showed the representation of the ACP loading factors of the Biolog ECO® samples (CLPP in the different samples).

The location in the sample plain (Fig. 4) and the loading factors (Fig. 5) is considered together. It is observed that in soil samples without effluent

irrigation, it is predominant the use of organic acids (D-galacuronic acid and hydroxybutyric acid) and one sugar (D-, L-glycerol phosphate) while in cultures irrigated with effluents are more linked to sugars and amino acids. Consumption of amino acids, such as L-asparagine, L-phenylalanine, L-threonine and sugars, such as D-mannitol or D-xylose, is remarkable in fertilized soils, including those with effluents.

These preferences are not exclusive; they only indicate the main sources.

Multivariant analysis shows consistent results with those obtained in kinetics and in diversity. The PCA shows a clear segregation between soils without irrigation and irrigated ones differentiating within this second group, the subscribers. The bacterial communities associated to each type of soil present a different consumption of carbon and nitrogen sources. The results of this study are consistent with those

found by Sofo et al. [38], who study olives under ecological and conventional management. These authors simply report differences in the consumption of sources depending on the type of exploitation, without discussing this result. Although it can only be speculated, since fertilization or the irrigation frequencies has not been analyzed, it seemed logical to think that the composition of organic matter added by these routes determined the functional structure of the studied communities. In this sense, Nautiyal et al. [39] indicate that the contribution of organic matter to the soil and the type of vegetation affects the functional structure of microorganisms in the soil.

In crops without exploitation (natural (A) and abandoned (B)), there is vegetation cover (herbaceous), while in other cases the spaces between vines and/or almonds or olive trees are free of vegetation cover. If the plant cover is larger, the release of compounds by exudation will also be, uniquely, organic acids [40]. In addition, absence of fertilization (in A and B) implies phosphorus poverty and it is known that plants exude organic acids to mobilize this nutrient [41].

On the other hand, the cover decomposition will lead to a higher bioavailability of sugars derived from cellulose and hemicellulose degradation [42], which is consistent with the results obtained in this work.

Finally, the enormous heterogeneity of sources consumed by the associated communities by fertilized and irrigated plants (C, H, G and F), is compatible with a high rate and diversity of exudation of these plants, among which components are mainly sugars and amino acids. Most probably the origin of the latter is the mineralization of proteins present in fertilizers.

4. Conclusions

It could be concluded from the study that:

(1) In all cases, anthropic exploitation of the soil entails a higher level of antibiotic resistance to abandoned soils or occupied by natural vegetation.

(2) Quinolones leave an ecological footprint in soil communities as even if anthropic pressure is removed, the resistance persists over time. However, the elimination of anthropic pressure could imply the reduction of the representation of strains resistant to tetracyclines, sulfamides, aminoglycosides and β -lactams.

(3) The kinetics of the consumption of carbon and nitrogen sources in bacterial communities studied is increased by anthropological effect. It cannot be ruled out from the fertilizer, the exudation of plants or the addition of bacteria through irrigation.

(4) Metabolic diversity of edaphic bacterial communities is lower in those soils without agrarian management. This is probably due to the addition of organic matter in the rest of the treatments than through the subscriber.

(5) The ACP indicates a clear segregation between the communities of unmanaged soil and the rest. In this second case, the authors' data showed more similarities with each other the samples subjected to soil fertilization.

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