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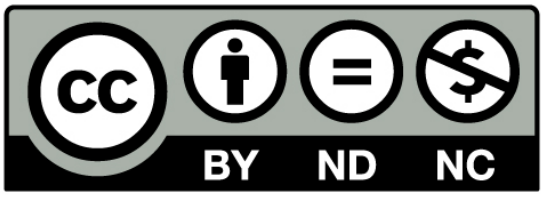

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Biofertilisers with olive-oil taste: isolation of plant growth-promoting rhizobacteria (PGPR) from "alperujo" compost

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HIGHLIGHTS

- "Alperujo" composts are an important source of N_2-fixing bacteria.
- There have been isolated and identified four PGPR bacteria in an "alperujo compost": *Burkholderia phymatum, Bacillus aryabhattai, Gluconacetobacter diazotrophicus* and *Pseudomonas stutzeri.*
- "Alperujo" composts are potentially biological fertilisers.

SUMMARY

Composting is a microbial technology which can be effectively used for transforming organic wastes into valuable organic amendments and fertilisers. Also, composts may be considered as biological fertilisers due to containing beneficial microorganisms capable of promoting plant growth and development. The aim of this research was the isolation of some plant growth-promoting rhizobacteria (PGPR) present in composts made of "alperujo", the main organic waste of the Spanish olive oil industry. We have focused in the nitrogen-fixing bacteria that were able or not to form symbiosis with legumes like soybean, common bean and chickpea. Also, free-living nitrogen-fixing bacteria and their ability to produce siderophores and solubilise insoluble soil phosphate were studied. We have found some bacteria that are able to nodulate common bean roots and we have identified four PGPR bacteria: *Burkholderia phymatum, Bacillus aryabhattai, Gluconacetobacter diazotrophicus* and *Pseudomonas stutzeri.*

INTRODUCTION (AND OBJECTIVE)

The world's population is rapidly growing and demands an increase of global food production [1]. Moreover, over the last 50 years, synthetic industrial fertilizers application (nitrates, ammonia, urea, etc.) has generated negative effects on the environment. Nutrient leakage has contaminated freshwater courses (surface and groundwater) and has provoked loss of soil biodiversity. Moreover, an increase in the emission of greenhouse gases from agriculture has been registered, which is directly related to global warming and climate change.

European Union has adopted a legislative initiative to promote sustainable development called "the Circular Economy" [2]. Its objectives are focused in reducing raw materials use, minimize waste production and encourage reuse and recycling. In Spain, olive oil industry generates a large amount of a solid by-product called "alperujo", a highly polluting organic waste that needs to be treated for its revalorisation [3]. In order to achieve that, composting can be performed due to it being a simple, inexpensive and effective method for transforming organic waste as "alperujo" (AL) into organic amendments and fertilisers [4]. The compost produced could also be used as a biological fertiliser

(biofertiliser). It may contain several microorganisms capable of promoting the development and growth of plants, although this issue has not been yet well documented.

Nowadays, it is well know that many bacteria can form beneficial associations with plants in their natural environments. It has been scientifically proven that plant growth-promoting rhizobacteria (PGPR) can improve development and growth of many plants. This is performed though several mechanisms such as biological control of pathogens, induced plant resistance, phytostimulation or increase of soil nutrients bioavailability.

In the latter, biological nitrogen fixation is an important mechanism that bacteria can use to transform atmospheric nitrogen gas (N_2) into ammonia using the nitrogenase enzyme. This process can be carried out through two ways, under symbiotic relationship with a certain group of plants such as legumes or under free-living conditions [5]. Other mechanisms are related to the production of iron chelators or siderophores and solubilisation of insoluble phosphate, both involved in increasing the availability of these soil nutrients for plants [6].

The aim of this research project was the isolation and identification of some bacteria with PGPR properties which are presented in AL composts. Namely, we have focused into their ability for fixing nitrogen, producing siderophores and solubilising insoluble soil phosphate.

MATERIALS AND METHODS

We have studied the presence of PGPR bacteria in a AL compost using two approaches: bacteria-legume symbiosis and free-living conditions. More information about agrochemical characteristics of the AL compost and the experimental PGPR procedures used in this study can be found in [4] and [6].

1. Bacteria-legume symbiosis

In order to elucidate if the AL compost used contained bacteria that were able to form symbiosis with legumes, two set of experiments were carried out. The legume plants used in this study were soybean, common bean and chickpea. They were selected due to their agronomic relevance. Seeds were surface-sterilized and germinated in petri dishes (10–12 seeds each) with 1 % agar (w/v) in darkness during 3-4 days at 30 °C. Selected seedlings were planted in hydroponic pots filled with vermiculite using the Leonard jar method (Figure 1), containing a mineral solution without any nitrogen source.

Figure 1. Leonard Jar system used in this study.

In the first experiment, solid AL compost (5 g per pot) was used and in the second one, liquid compost was added (1mL per seed). Liquid compost was obtained using a 1:20 solid to liquid ratio during 2 h of mechanical extraction. Both experiments consisted in two treatments, with and without compost, and 3 replicates per treatment were performed.

The experiments were carried out under sterilized conditions during 3-4 weeks at the facilities of Greenhouse and Growth Chamber Service of Estación Experimental del Zaidín (EEZ).

2. Free-living conditions

2.1. Liquid compost and isolation of N_2-fixing bacteria

Liquid compost was obtained by mechanical extraction using 1:20 solid to liquid ratio for 2 h with sterile saline solution (NaCl 0.9%). After that, serial dilutions were prepared up to 10^{-6} . Petri dishes containing Burk medium (specific medium for isolation of N_2-fixing bacteria without any nitrogen source) were inoculated. 30 µL of each dilution were added and extended using a Drigalsky spatula. Cultures were incubated for 4 weeks in darkness at 30 ºC. This process was duplicated.

2.2. Selection of bacteria

Bacteria who showed different morphology (color and shape) were selected using a magnifying glass and a light microscope. They were cultured separately in a new petri dish containing Burk medium and incubated at 30 °C for several weeks. Trace N in the media was prevented by using high purity products. Once grown, the isolates which showed a better growth were re-grown in Burk liquid medium at 30 °C for 1 week. For each isolate, two tubes were prepared. One of them was for the isolation of DNA and the remaining for PGPR tests.

2.3. Plant growth-promoting properties (PGPR): siderophore production and solubilization of inorganic phosphates.

In order to check the PGPR properties of the N_2-fixing strains isolated, 3 mL of each bacterial culture was used to inoculate Petri dishes containing two specific media for qualitative PGPR analysis:

- Phosphate solubilisation: the culture medium contained insoluble tricalcium phosphate. Solubilisation of phosphate was considered positive if a clear halo was formed around the colony.
- Production of siderophores: the culture medium contained a blue chromogenic compound. Siderophore production was considered positive if an orange halo was formed around the colony.

The cultures were incubated for two weeks in darkness at 30 °C. Next, halo diameters and colony sizes were recorded. The phosphate and siderophore efficiency (E) of each strain was determined by the formula: [E = diameter of PGPR activity / diameter growth x 100]. E data were then refereed to *A. brasilense* C16 as a PGPR standard (100%).

2.4. Identification of isolates

2.4.1 Isolation of DNA and amplification of the 16S rRNA gene

For DNA extraction and PCR amplification, genomic DNA was isolated from bacterial cells using the Real Pure Genomic DNA Extraction kit (Durviz, Spain), according to the manufacturer's instructions. Quantity of DNA was determined using a Nanodrop spectrophotometer (NanoDrop ND1000, Thermo Fisher Scientific, USA). To identify the selected microorganisms, PCR amplifications of 16S rRNA gene fragments were performed using the two universal opposing primers, fD1 and rD1, which amplify conserved sequences [7].

2.4.2 Electrophoresis

To check the results from PCR amplification, electrophoresis technique was used. This technique allowed to separate molecules according to their mobility in an electric field. 5 µL from the PCR reaction were run in a 0.7% agarose gel in TBE buffer at 90V. The gel was stained GelRed™ (Biotium) and visualized under UV light.

2.4.3 Sequence analysis

PCR products were purified and sequenced in the Sequencing Service available in the Experimental Station of Zaidín (EEZ-CSIC). The sequences obtained were compared with the metagenomic bacteria database EzTaxon available in http://www.ezbiocloud.net/eztaxon.

RESULTS AND DISSCUSSION

Biological N_2-fixation by bacteria-legume symbiosis.

Although N_2 constitutes around 80% of the atmosphere, fixed nitrogen is a limiting nutrient in most environments. N_2 cannot be directly assimilated by plants, but it becomes available through biological fixation, a process carried out exclusively by prokaryotes.

In the first plant experiment, no nodules were recorded in soybean, common bean and chickpea roots when solid AL compost was added. On the other hand, in the second experiment, nodules were found only in common bean roots when liquid compost extract was used (Figure 2).

These results did not seen to be representative of the symbiotic nitrogen fixation for no differences between plants growth with and without AL compost were found (Figure 3). More experiments need to be carried out to elucidate if N_2-fixing bacteria, present in AL compost, are able to nodulate common bean or not.

Figure 2. Nodulated roots of common bean.

Figure 3. Soybean, common bean and chickpea plants treated with and without AL compost.

***In vitro* plant growth-promoting traits**

Biological N_2-fixation presents considerable interest among PGPRs properties and, accordingly, it was set as a priority in the search for PGPRs isolated from the AL compost. The culture dependent approach was used in this study to isolate N_2-fixing bacteria from the AL compost. A total of 38 strains with *different morphology (color and size) (Figure 4 and 5) were selected but only* 9 were derived *to PGPR tests.*

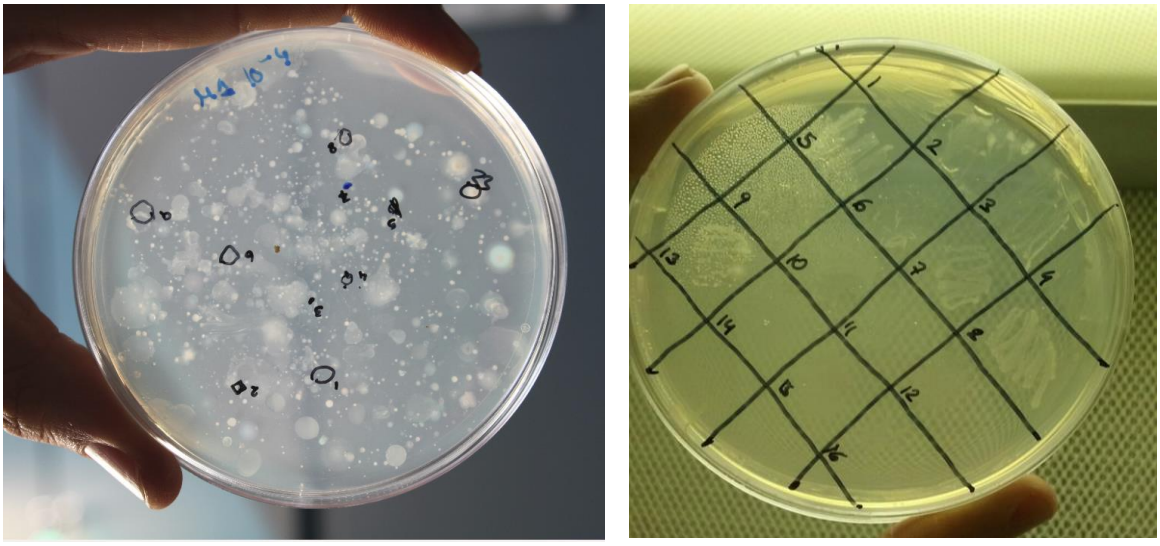

Figure 4. Cultivable N_2-fixing bacteria isolated from AL compost.

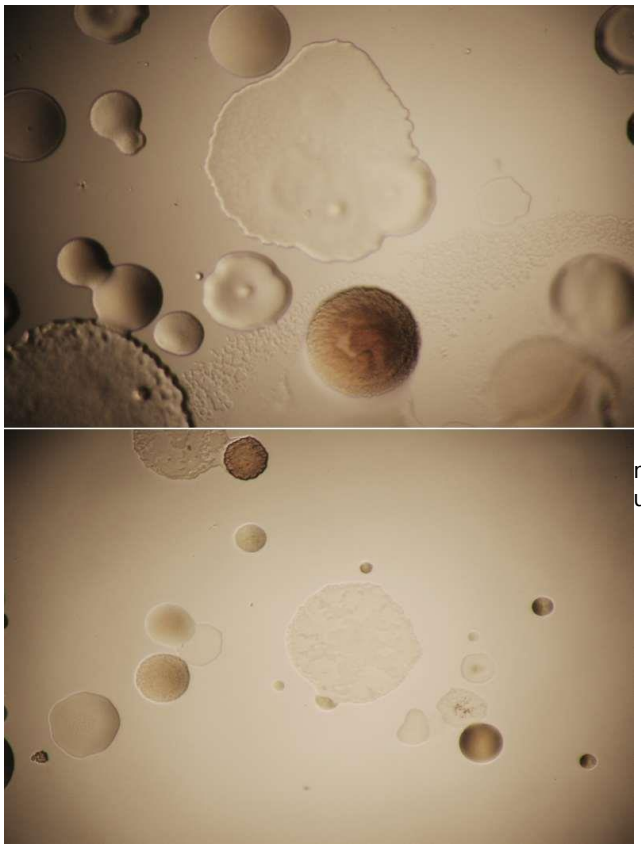

Figure 5. Example of bacteria with different morphology (color and shape) selected in this study using a magnifying glass and a light microscope.

The cultivable N_2-fixing bacteria presented in the AL compost used were counted using serial dilution protocol. *1.62 x 10^8 of colony-forming units (CFU) per dry gram of compost were obtained.*

Four strains (1, 2, 7 and 9) were able to produce siderophores and also, to solubilize mineral phosphate (Table 1 and Figure 6). In general, all strains showed higher E values compare to the PGPR reference strain A. brasilense C16. The highest values of siderophores corresponded to strain 2 (150 %), and phosphate solubilisation to strain 7 (290 %) respectively.

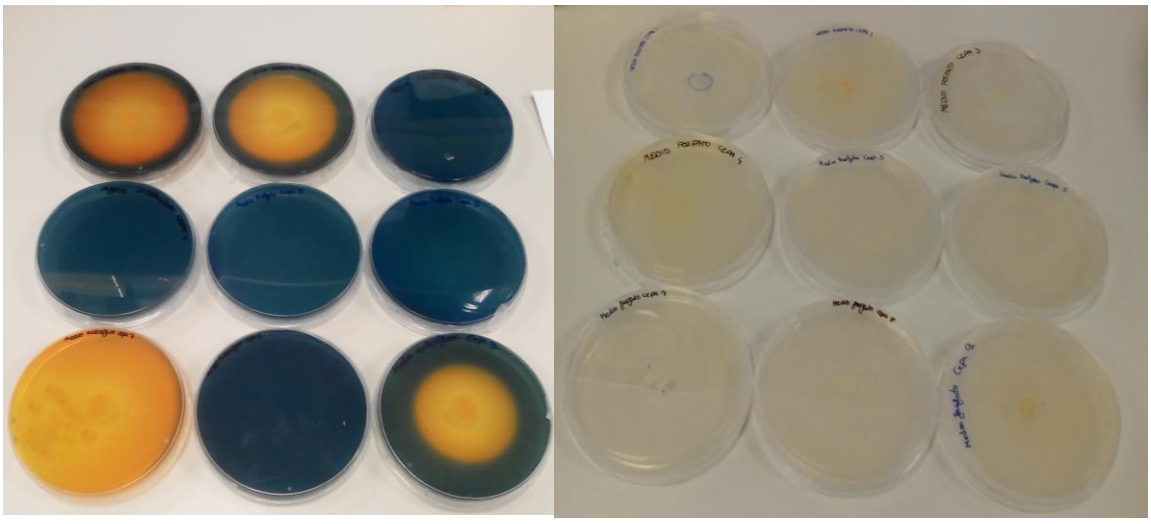

Figure 6. Results of PGPR tests of siderophore production (left) and mineral phosphate solubilizing ability (right) from the 9 bacterial strains isolated from the AL compost.

Identification of N_2-fixing rhizobacteria

In order to identify up to genus/species level the four bacteria that showed better PGPR properties, amplification of 16S rRNA gene were carried out (Figure 7). The nearly complete 16S rRNA gene

sequence clustered the 4 strains into 4 different genera (Table 2). Ez-Taxon pairwise alignments between globally aligned type sequences showed they were closely related to members of genera *Burkholderia phymatum* STM815^T (strain 1), *Bacillus aryabhattai* B8W22^T (strain 2), *Gluconacetobacter diazotrophicus* PAL^T (strain 7) and *Pseudomonas stutzeri* ATCC 17588^T (strain 9). It has been previously demonstrated that these genera were able to solubilize phosphate and produce siderophores [6, 8, 9 and 10].

Table 1. Results of PGPR tests of siderophore production and mineral phosphate solubilizing ability.

	Siderophore production			
Strains	**Did it form halo?**	**Colony diameter (cm)**	**Halo diameter (cm)**	**Efficiency* (%)**
1	YES	0.7	1.4	125.0
2	YES	0.6	1.8	150.0
3	NO	-	-	-
4	NO	-	-	-
5	NO	-	-	-
6	NO	-	-	-
7	YES	0.8	1.5	93.8
8	NO	-	-	-
9	YES	0.7	1.4	100.0
	Mineral phosphate solubilizing ability			
Strains	**Did it form halo?**	**Colony diameter (cm)**	**Halo diameter (cm)**	**Efficiency* (%)**
1	YES	2.0	7.3	182.5
2	YES	2.0	6.7	176.3
3	NO	-	-	-
4	NO	-	-	-
5	NO	-	-	-
6	NO	-	-	-
7	YES	1.5	8.8	290.0
8	NO	-	-	-
9	YES	1.8	5.1	141.6

* these data were obtained using *A. brasilense* C16 as a PGPR reference

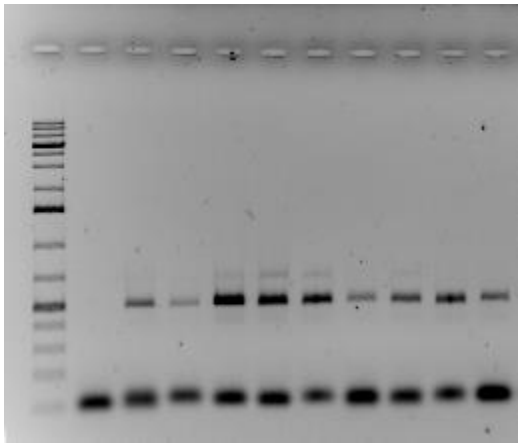

Figure 7. Electrophoresis of 16S rRNA gene PCR amplifications of the isolated bacterial strains.

Table 2. Identification of PGPR strains isolated from AL compost

Strains	Closest relative species according to 16S rRNA gene sequence	Similarity (%)
1	*Burkholderia phymatum STM815*	99.7
2	*Bacillus aryabhattai B8W22*	100.0
7	*Gluconacetobacter diazotrophicus PAL*	100.0
9	*Pseudomonas stutzeri* ATCC 17588	88.2

CONCLUSIONS

1. We did not detect any bacteria with the ability to nodulate soybean or chickpea plants.
2. We detected some bacteria that were able to nodulate common bean roots, but more experiments are needed to confirm this result.
3. The presence of cultivable nitrogen-fixing bacteria in the AL compost used was relevant (*1.62 x 10^8 CFU per dry gram of compost)*.
4. We have isolated and identified four bacterial strains with PGPR properties: *Burkholderia phymatum, Bacillus aryabhattai, Gluconacetobacter diazotrophicus* and *Pseudomonas stutzeri.*

ACKNOWLEDGEMENTS

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MY OWN IDEAS

Elena Navarro García

Al comenzar este proyecto no sabía muy bien cómo se iba a desarrollar y no estaba segura de si no me arrepentiría de participar en él, pero con el paso del tiempo y de las sesiones me ha ido gustando cada vez más y pienso que es una experiencia que debería vivir todo aquel que se sienta atraído por la ciencia.

La razón principal por la que pienso de esta manera es debido a que soy una firme defensora del aprendizaje práctico, es decir, en mi opinión, los conceptos se afianzan mucho mejor si ves o haces una prueba de ello. Gracias a este proyecto he podido poner en práctica muchos de los conceptos aprendidos anteriormente en mi instituto ya que, debido a la falta de tiempo, no solemos hacerlo y aquí nos ha dado la oportunidad de ello.

María Molina Muñoz.

Cursábamos cuarto de la ESO cuando nuestra profesora Soledad de física y química entró en la clase con una noticia que cultivó intriga e interés en todo el aula. La oportunidad de poder participar en un proyecto de investigación como tal, con científicos, medios y las instalaciones adecuadas era posible. El CSIC, la Universidad de Granada y los proyectos PIIISA nos brindaban esta oportunidad: mutación de bacterias, cúmulos estelares, el genoma humano, biología tumoral, jardín microscópico, agujeros negros… Infinidad de temas y proyectos estaban a solo unos pequeños pasos. Desafortunadamente, tanto para el resto de mis compañeros como para mí, las plazas se agotaron antes de que nuestra profesora pudiera apuntar ningún nombre, pese a su esfuerzo, las oportunidades cesaron a tiempo récord. Llegado el 2016, nuevo año, nueva vida y nuevas ocasiones de participación. Fue entonces cuando por fin se me ofreció el disfrute de este proyecto. Sinceramente, una de mis mejores experiencias como estudiante. Todavía recuerdo como el ayer, el primer día que llegamos al CSIC, nuestra profesora nos invitó a desayunar y nos deseó suerte para el resto del día. Quedamos todos los alumnos reunidos en un gran salón de actos, recuerdo también girar mi cabeza varias veces para contemplar con intriga la sala, cuando vi a dos investigadores sentados en las sillas del fondo. Quién me iba a mí a decir, que estos dos jóvenes se convertirían en mis investigadores, en los propulsores de tanta ilusión y gusto: Germán Tortosa y Antonio Castellano.

Bajo el proyecto: ''Biofertilizantes con sabor a aceite de oliva" y las puertas del laboratorio 202 un pequeño equipo de seis alumnos de diferentes centros estaría trabajando al unísono durante varios meses como verdaderos científicos: y es que nuestros coordinadores nos vistieron con una bata blanca, guantes y nos dejaron, como bien dice Germán ''cacharrear'' con todo el material científico, ''eso es lo que a ellos les gusta, cacharrear''. Pudimos usar lupas, microscopios ópticos, programas de identificación de bacterias, cabinas de flujo laminar, pipetas. ¡Cultivamos en casa nuestras propias bacterias y dos macetitas de soja o garbanzo fueron regadas por nosotros mismos! Alucinante.

El trato de ellos hacia nosotros, fue grandioso. Todas las sesiones nos invitaban a desayunar, nos presentaban a sus compañeros de laboratorio, resolvían dudas e incluso varias fotos y vídeos nos eran sacadas.

Realmente esta experiencia ha alcanzado parámetros de ilusión que no pensé que fueran posible. He vivido cada sesión con tanta ilusión que dudo mucho que se me vaya a olvidar fácilmente, he disfrutado tanto viendo crecer a nuestras cepas, aislando su ADN y poniéndoles nombres que el deleite ha sido máximo. En definitiva me sentía grande dentro de mi pequeño cuerpo, visualizaba mi futura profesión a cada paso del procedimiento. Me sentía realmente parte de esta gran comunidad científica y aunque mis inseguridades eran notables; pues nunca antes había usado una pipeta ni inoculado placas Petri, la confianza y paciencia de nuestros investigadores fue de gran ayuda.

Así que no me queda más que agradecerles a ellos y a los coordinadores de esta actividad, por recibirnos con los brazos tan abiertos, por su energía y su capacidad transmisora de amor a la ciencia, por su apoyo altruista e iniciativa y por convertir en verídico este proyecto haciendo felices a tantos pequeños científicos por unos pocos días.

Laura Palma Pérez.

Cuando me dijeron que me había tocado este proyecto pensé que iba a estar bien pero sinceramente antes de haberlo hecho hubiera preferido entrar en el de genética, pero desde el primer día que pisamos el laboratorio supe que este proyecto era el mejor de todos porque en comparación con

otros compañeros de otros proyectos, nosotros hemos estado prácticamente todo el rato en el laboratorio. Por otra parte la relación con los compañeros de proyecto ha sido muy buena y desde el primer día nos hemos llevado muy bien y no hemos tenido ningún problema en ayudarnos entre nosotros. Gracias a este proyecto he podido descubrir cómo se trabaja de verdad en un laboratorio, y la verdad que me ha dado muchas ideas a la hora de elegir una carrera para el futuro.

Mª Carmen Sarmiento Vega.

En conclusión, esta experiencia me ha encantado. Es impresionante ver el lugar en donde grandes investigadores trabajan a diario para ayudar al mundo. Lo que más me ha gustado ha sido poder trabajar con los aparatos específicos de investigación, pero sobretodo el microscopio. Este proyecto me ha servido muchísimo para saber que es trabajar en equipo y el esfuerzo que requiere descubrir cualquier cosa por pequeña que sea.

Carlos Ortega Fernández.

La impresión que he tenido sobre este proyecto ha sido muy gratificante debido a que he podido experimentar como es el trabajo en un laboratorio utilizando todos los instrumentos que eran necesarios para realizar nuestro proyecto relacionado con el compost. La parte que más me ha gustado del proyecto fue cuando miramos las bacterias a través del microscopio electrónico y luego hicimos una placa master con las bacteria que fuimos seleccionando según su morfología para luego crecerlas y extraer su ADN. Esto puede que no me haya servido de nada por ahora, pero estoy seguro de que en un futuro me será muy útil ya sea para decidir la carrera que quiero estudiar o para cualquier otra cosa.

Alba Díaz Arco.

Este proyecto me ha encantado ya que está relacionado con la ciencia y sobre el medio ambiente, de gran importancia para nuestro futuro. Al haber tratado con diversas prácticas y haber aprendido cosas sobre este tema por primera vez he aprendido como se trabaja en un laboratorio ya que en mi instituto lo utilizamos pocas veces. También me ha ayudado a saber si realmente me gustaría trabajar en estos ya que si no pruebas algo no sabes realmente como es, como suele pasar cuando tienes expectativas. Es muy importante este tipo de proyectos por esto mismo ya que muchas personas cambian de carrera porque eso no es lo que pensaban o tenían otra idea de ello, de esta manera podemos evitarlo.