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Analysis of vitamin C content of fruits from five pepper varieties showing different pungency levels and antimicrobial potentiality of capsaicin

Paula Entrena-Aranda[1*], Marta Roldán-Martín[1*], Ignacio Mengíbar-López[1], Laura Rodríguez-López[1], Ainhoa Yolanda Moreno-Duperón[1], Elena De la Torre-Amat[1], Julia Shan Vida-Lara[1], Antonio Quesada[1#], Carmelo Ruiz[2], María Jesús Campos[2], José Manuel Palma[2#]

[1]*IES Zaidín-Vergeles. Primavera, s/n, 1808 Granada, Spain.*

[2]*Department of Biochemistry, Cell and Molecular Biology of Plants. Estación Experimental del Zaidín. CSIC. Profesor Albareda 1, 18008 Granada, Spain.*

**These authors equally contributed to this work*

#Corresponding author: quesadaramos@gmail.com; josemanuel.palma@eez.csic.es

Highlights:

- *A simple and easy method has been developed to determine vitamin C (ascorbate) in a High School Laboratory. The method provides data which correlate with results obtained from high accuracy procedures.*
- *A correlation between ascorbate content and pungency level in pepper fruits has been detected.*
- *Capsaicin seems to exert inhibitory effect in the growth of the bacterium Bacillus subtilis.*

Summary

In this work, the nutritional properties and antimicrobial activity of five pepper varieties, including dulce italiano (bell pepper), boiro (a Padron-type pepper), green jalapeño, red chilli, and habanero, have been investigated. The dry matter provided by each of these varieties was analyzed, and their caloric content were withdrawn from searches in databases. Thus, it was found the following caloric contents: Habanero and red chilli provided 40 kcal/100 g; jalapeño green, 29 kcal/100 g; dulce italiano, 23 kcal/100 g; and boiro (padrón), 21 kcal/100 g. On the other hand, an easy and simple method was set up to determine vitamin C (ascorbic acid, ascorbate) in crops, using lugol and starch. The data provided by the application of this method indicated that red chili was the variety with the highest vitamin C content, and boiro the one with the lowest. The antimicrobial activity of both crude extracts from pepper fruits and pure capsaicin was probed against five bacterial species: *Escherichia coli*, *Bacillus subtilis*, *Xanthomonas campestris, Dickeya dadantii* and *Ralstonia solanacearum*. Solid (Petri dishes with agar) and liquid media were used for the assays. Our data indicated that capsaicin is able to inhibit the growth of *B. subtilis*, but not that of *E. coli*, perhaps this latter case due to the great adaptation of this species to environmental conditions.

Keywords: antibacterial, ascorbate, *Bacillus subtilis*, *Capsicum annunm*, capsaicin, *Dickeya dadanti, Escherichia coli*, pepper, pungency, *Ralstonia solanacearum, vitamin C, Xanthomonas campestris.*

INTRODUCTION

Pepper (*Capsicum annuum* L.) is an annual herbaceous plant which belongs to the Solanaceae, a family that also includes a number of important crops for the human diet, such as tomato (*Lycopersicum esculentum*), potato (*Solanum tuberosum*), and aubergine (*Solanum melongena*) [1,2]. With respect to pepper fruits, a colorful assortment of varieties can be found, displaying bright red, yellow, orange, purple and others colors once the fruits ripen. Besides their appearance, pepper fruits are also differentiated according to their organoleptic features and culinary uses. Thus, hot and sweet pepper fruit varieties can be found with a diversity of local names [3-5]. The great versatility of pepper fruits in terms of culinary uses and the considerable increase in production over the last years justifies the interest and economic importance of this crop species.

Due to their high content in water, pepper fruits are low in calories and are a good source of antioxidants such as vitamin C (ascorbic acid, ascorbate), vitamin A (β-carotene as a precursor), flavonoids, polyphenols, capsaicinoinds and diverse minerals (K, Fe, Mn, Cu, P) [6,7]. In fact, 50–80 g of sweet pepper fruits are sufficient to provide 100% and 25% of the recommended dietary allowance (RDA) of vitamin C and A, respectively [8–13]. Vitamin C, which might be considered as the paradigm of the low molecular weight antioxidants, is essential in animals and humans since it participates in many metabolic pathways and in the prevention of a number of pathologies [14-17]. Vitamin A is obtained once β-carotene breaks down symmetrically, so each carotene molecule gives rise to two vitamin A molecules. Vitamin A is important in the regulation of redox homeostasis in diverse physiological events related to vision, growth and development, among others [17–21].

Capsaicinoids are specialized metabolites produced exclusively by the genus *Capsicum*, responsible for the pungency of hot pepper fruits [22]. This is a family of about 23 compounds where capsaicin and dihydrocapsaicin are the two main chemicals accounting for about 95% of total capsaicinoids [23]. Special attention has been paid to capsaicin, which has been carefully studied for its analgesic, antioxidant, anti-inflammatory, and anti-obesity properties [24-26]. From an evolutionary point of view, capsaicin is originally important for avoiding bitting by insects and other animals. Pungency is measured by the Scoville scale, expressed as Scoville Heat Units (SHU). Bell peppers are not pungent (SHU=0), while some varieties can reach to more than 2 million SHU. In this scale, the highest level, 16,000,000 is assigned to pure capsaicin. Consequently, the metabolic and biochemical content of *Capsicum* species is not only valuable for the plant itself, but also for human health due to its potential use in diverse therapies according to the biological activity.

There are evidences about the beneficial effects of pepper intake. Chopan and Littenberg [27] demonstrated that the consumption of hot red chilli peppers was associated with a 13% reduction in the instantaneous hazard of death. In a study carried out in an adult

Mediterranean population, it was concluded that regular consumption of chili pepper is associated with a lower risk of cardiovascular disease [28].

Besides the pharmacological and nutritional properties mentioned above, capsaicin has also attracted interest in Microbiology due to its antimicrobial activity. Several studies have demonstrated the bactericidal *in vitro* effect of capsaicin against *Helicobacter pylory* [29], food borne pathogens [30] , or *Streptococcus pyogenes*, a major human pathogen [31]. Regarding the antimicrobial mechanism of action, it has been suggested that capsaicin probably exerts its bactericidal effect through membrane damage and it has been reported that this molecule also prevents biofilm formation [32]. Consequently, ingestion of chilli peppers, rich in capsaicin, could have a protective effect against microorganisms capable of causing infections in humans.

Thus, with this background on the relevance of pepper fruits as important reservoir of vitamin C and their use as potential microbial tools, in this work we aimed at developing a quick and easy method to determine ascorbate content in five pepper fruit varieties with different pungency levels, but applicable to analyze other fruits in a Secondary School laboratory. Additionally, the potential microbial activity of capsaicin, an alkaloid exclusive of the genus Capsicum was investigated using three bacterial species as targets.

MATERIALS AND METHODS

Pepper fruits

Five varieties of peppers were used for this work: Dulce italiano (bell pepper), boiro (a Padron-type pepper), green jalapeño, red chilli, and habanero. All of them were obtained from the local markets (**Figure 1**).

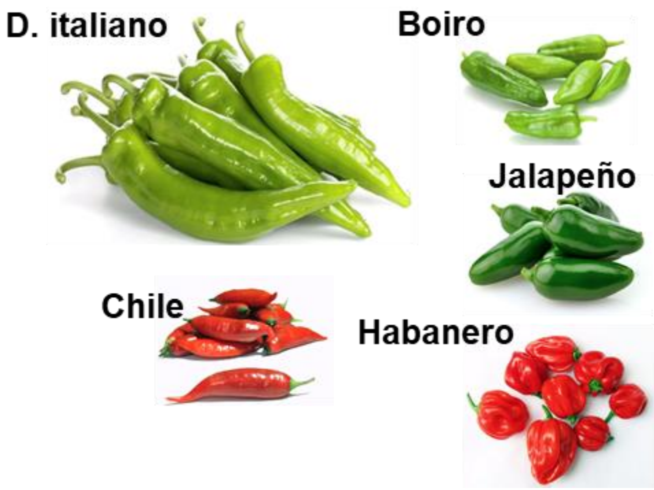

Figure 1. Five pepper fruit varieties used in this work.

Bacterial species

Two groups of microorganisms were used to detect potential antimicrobial activity. On the one side, *Escherichia coli*, a gram negative species that normally lives in the human and animal intestine, and *Bacillus subtilis*, a gram positive bacterium found in soil and the gastrointestinal tract of ruminant and humans. On the other side, three plant pathogenic gram negative bacteria: *Xanthomonas campestris*, *Ralstonia solanacearum* and *Dickeya dadanti*. All the microorganisms were provided by the group of Environmental Microbiology and Biodegradation, Estación Experimental del Zaidín (CSIC), Granada, Spain.

Dry matter assay

Lots of fresh pepper fruits were weighed and then oven-dried at 50ºC for a week. Then, samples were weighted again and the loss of water was calculated by subtraction. Dry matter was expressed as the percentage with respect to the original fresh weight value.

Vitamin C determination

Vitamin C (ascorbic acid, ascorbate) content of peppe fruits was evaluated by a titration method with a lugol solution and starch as indicator. Basically, iodine of the lugol solution is able to react with starch resulting in a dark blue colour. Ascorbic acid prevents the appearance of such colour as it reacts with iodine. The higher concentration of ascorbic acid, the higher volume of the lugol solution to stain starch is necessary.

A standard curve was prepared as follows. Test tubes with 2 ml of starch 0,5% (w/v) were added 1 ml of ascorbic acid solutions of known concentrations. With the help of a micropipette, increasing volumes of the lugol solution were added until the appearance of the dark blue colour. The concentration on the known ascorbate concentrations were plotted against the lugol volumes used.

For the determination of ascorbate in samples, 10 g of pepper fruits were ground in a mortar with a pestle with 10 ml of distilled water. The homogenate was filtered with the help of nylon-cloth until clean homogenates were obtained. Then, 1 ml of homogenates was added to 2 ml of 0,5% (w/v) starch solution and evaluated with the procedure described above. Volumes of lugol used were then extrapolated to the standard curve. An average of three measures were used for calculation of vitamin C concentration in peppers.

Antimicrobial activity evaluation

Pepper extracts preparation. Pepper fruits were dried at 50ºC for a week. The dry matter was crushed with the help on a mortar and a pestle. Then, 2 g of the dried material were dissolved into 10 ml of 70% (v/v) ethanol for a week at room temperature. The mixture was filtered and stored at -5ºC in bottles until use.

Agar disk-diffusion method. Twenty mililiters of TSA (triptone soya agar) medium were poured into sterile Petri dishes as a basal medium. Likewise, 5 ml of semisolid medium [triptone soya broth + agar 0,6% (w/v)] at 42ºC were inoculated with 100 microliters of liquid culture of the bacteria to be tested and spread over the basal solid medium. Agar plates were then incubated for 24 hours. Sterile Whatman filter paper disks (6 mm diameter) were soaked with 25 μl of pepper extracts, let dry on a sterile plastic grid and deposited on the inoculated plates. For methanol negative control, blotting paper disks were dipped into ethanol 70% (v/v). For positive control, paper disks were impregnated with *Punica granatum* (pomegranate) peel extracts whose antimicrobial action was determined in our laboratory with this method [33]. Estimation of the inhibitory activity was made measuring the size of the inhibition halo.

Agar well-diffusion method. The agar plate was prepared and inoculated as before and a hole with an 8 mm diameter was punched aseptically with a sterile pipette. Pepper extracts (50 μl) were deposited into the wells and incubated for 24 hours. Estimation of the inhibitory activity was made by measuring the extension of the inhibition haloes.

Antibacterial liquid inhibition assay. Two batches of test tubes with 5 ml of sterile TSB (Triptone soy broth) medium were inoculated with 20 μl of liquid cultures in stationary phase, one with *Escherichia coli* and the other batch with *Bacillus subtilis*. Then, 50 μl of either each pepper extract or capsaicin 0,05 M, prepared in ethanol, were added to the test tubes. One set with no treatment at all was used as a positive control and reference of the optimal growth. Then, they were incubated at 28ºC for 20 hours. Growth was estimated measuring the optical density at 600 nm in a Shimadzu UV 120-02 spectrophotometer.

RESULTS AND DISCUSSION

Five varieties of peppers with different levels of pungency have been studied in order to know their nutritional and healthy properties. Information about pungency was obtained from database on the Internet site https://www.pepperscale.com/hot-pepper-list/. The five varieties were ranged according to their increasing pungency level in the Scoville scale as follows: Dulce italiano (bell pepper): 0 SHU; boiro: 1.500 SHU; jalapeño green: 5.250 SHU; red chilli: 22.500 SHU; habanero: 325.000 SHU.

Dry matter and nutritional value

One of the most important features related to nutritional value of peppers is their water content, since this is a fruit which provides very low calories in the diet. Thus, the nutritional value of peppers was studied from database on Internet sites. Habanero and red chilli provided 40 kcal/100 g; jalapeño green, 29 kcal/100 g; dulce italiano, 23 kcal/100 g; and boiro (Padrón), 21 kcal/100 g [https://www.nal.usda.gov/fnic/usda-nutrient-data-laboratory]. Likewise, the dry matter of the five varieties was evaluated. Results are shown in **Table 1**. The variety with the highest water content of water was dulce italiano (97,4%); on the contrary, red chilli displayed a 74,8% of water. Boiro, green jalapeño and habanero ranged between 88,4% and 92,6%.

Table 1. Dry matter of the five studied peppers varieties.

Variety	Fresh weight (g)	Dry weight (g)	% Dry weight
Dulce italiano	196,7	5,2	2,64
Boiro	99,3	8,2	8,26
Jalapeño Green	89,1	6,6	7,41
Chile Red	50,8	12,8	25,19
Habanero Red	80,1	9,3	11,61

Vitamin C content of fruits from the five varieties

Vitamin C is an important antioxidant present in peppers [17]. The level of vitamin C in the fruits of the five studied pepper varieties was analyzed. To begin with, a standard curve was prepared with known amounts of ascorbic acid using a titration with lugol as described above (**Figure 2**). A very good correlation was found, so this method seems to be appropriate to be used in a High School laboratory for training of students.

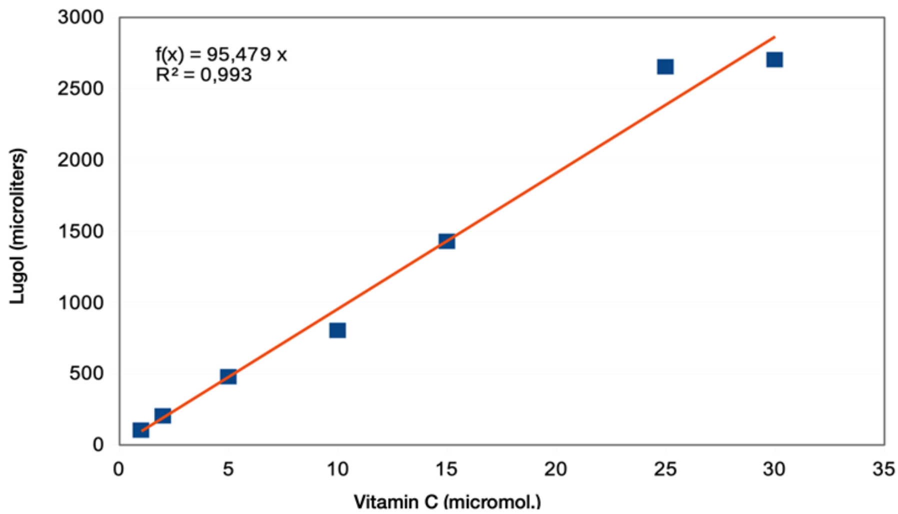

Figure 2. Standard curve for vitamin C determination.

Then, the ascorbate content was determined in samples and titration data were extrapolated in the above standard curve. **Table 2** shows the average volume of lugol used for each titration, the concentration of ascorbic in the extracts and the quantity of vitamin C (mg) per g of fresh pepper in the five pepper varieties analyzed. The varieties with the highest vitamin C concentration were red chilli pepper, habanero and bell pepper. On the contrary, our boiro pepper samples showed the lowest concentration of ascorbic acid. According to these data, any person would need an intake of 80,65 g of red chilli pepper to reach the recommended daily allowance (RDA) of vitamin C.

Table 2. Determination of vitamin C in pepper extracts from fruits of five pepper varieties. The intake amount of each type or pepper to reach the recommended daily allowance (Vitamin C RDA: 80 mg) is also included.

Variety	Lugol (microliters)	Ascorbic acid (mM)	Vit C mg/g fresh weight	Pepper intake Vitamin C RDA
Bell pepper	316,67 ± 20,41	3,32	0,585 ± 0,038	136,75 g
Boiro	50,00 ± 0,00	0,52	0,091 ± 0,00	879,12 g
Jalapeño Green	133,33 ± 4,08	1,40	0,247 ± 0,008	323,89 g
Red Chilli	537 ± 17,67	5,63	0,968 ± 0,07	80,65 g
Habanero	316,67 ± 20,41	3,32	0,585 ± 0,038	136,75 g

The relationship between pungency and vitamin C concentration was investigated in this work (**Figure 3**). Except for dulce italiano and red chilli varieties, a direct correlation between ascorbate concentration and pungency was observed: thus, the higher concentration of vitamin C, the higher concentration of capsaicin (pungency, SHU values). This pattern matches with a previous report where a role for ascorbate to protect capsaicin metabolism was proposed [4].

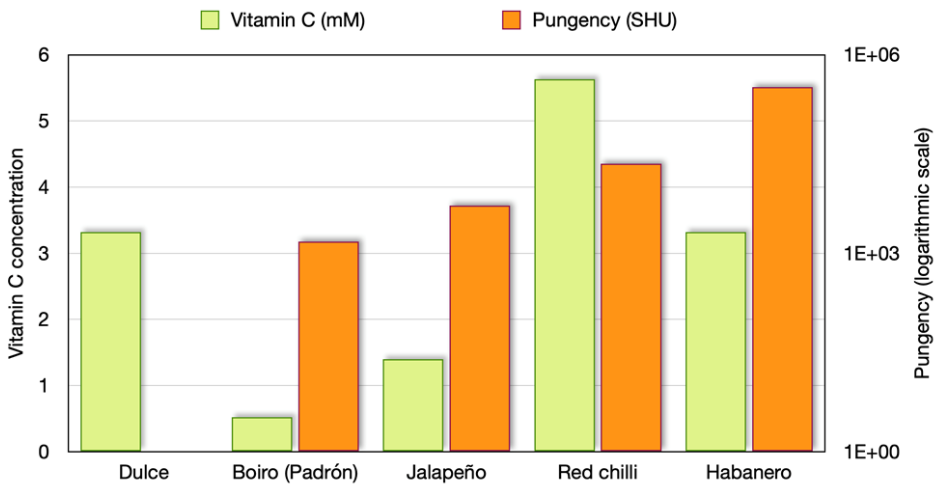

Figure 3. Relationship between pungency (measured in Scoville Heat Units, SHU) and vitamin C content in the five pepper varieties investigated.

Antimicrobial properties of pepper fruits

The antimicrobial effect of pepper extracts from several varieties has been described such it occurs with bell pepper [34], jalapeño [35] and chilli pepper [30]. The potential antimicrobial activity of our extracts was tested with the agar disk difussion method. None of our varieties showed inhibition halos against *E. coli*, *B. subtilis*, *X. campestris*, *R. solanacearum* or *D. dadanti*. (**Figure 4**). No bactericidal activity was found either in plates with disks soaked in an ethanolic solution of capsaicin with the following concentrations: 50 mM, 25 mM, 12,5 mM, 6,25 mM, 3,1 mM and 1,6 mM. On the contrary, control plates with pomegranate peel extracts gave positive results (**Figure 5**).

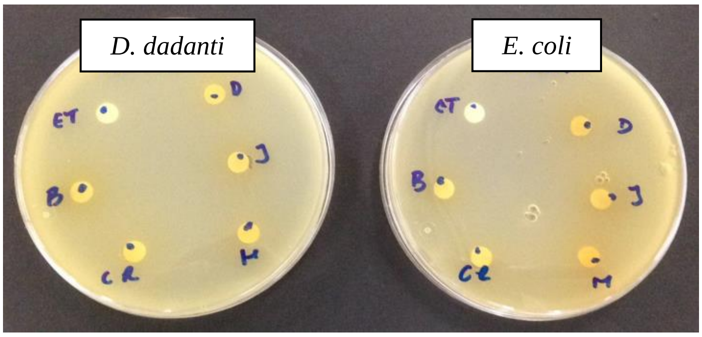

Figure 4. Absence of inhibition halos of pepper extracts against *D. dadanti* and *E. coli*.

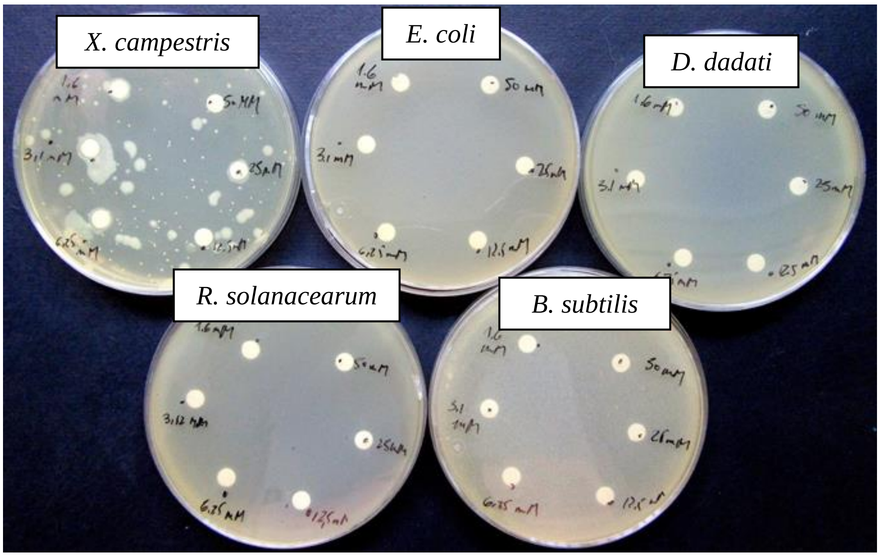

Figure 5. Negative results of the inhibition test with capsaicin in five bacterial strains: *X. campestris*, *E. coli*, *D. dadanti*, *R. solanacearum* or *B. subtilis*.

With the exception of boiro pepper extracts against *X. campestris*, similar results were obtained with the agar well diffusion method. Thus, no inhibition halos were observed, either with pepper extracts or under the ethanolic solutions of capsaicin with the mentioned concentrations (**Figure 6**).

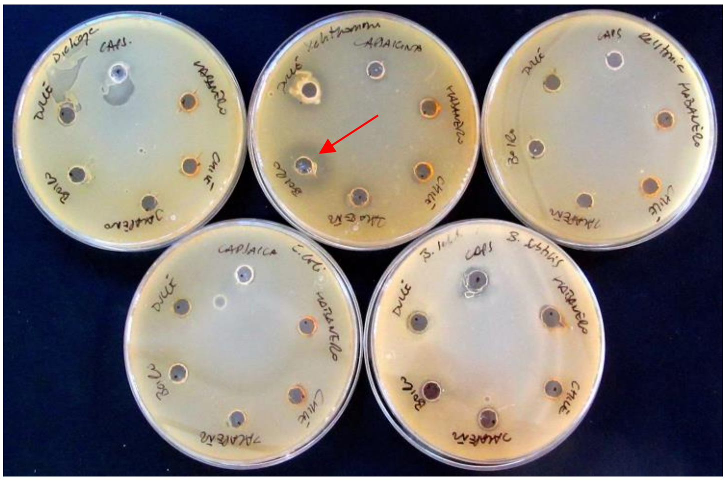

Figure 6. Agar well diffusion test. Negative effect of capsaicin and pepper extracts (except boiro extract against *X. campestris*, see red arrow) were observed.

Experiments with liquid medium were also carried out, but only with *E. coli* and *B. subtilis*, displaying different results (**Figure 7**). Thus, whereas *E. coli* was not affected by capsaicin 50 mM, and showed very little inhibition with pepper extracts, the growth of *B. subtilis*, estimated by the optical density of cultures, was significantly limited by the presence of capsaicin (45,5%) and pepper extracts: bell pepper (70,7%), boiro (74,4%), jalapeño green (76%), red chilli (63,5%) and habanero (78,4%).

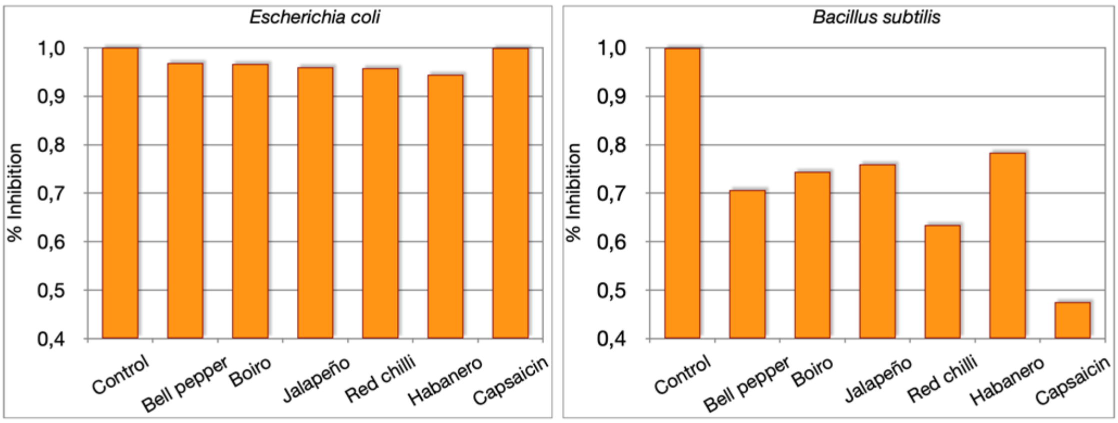

Figure 7. Results of the inhibition tests in liquide cultures of *E. coli* and *B. subtilis*.

It has been previously proved that capsaicin inhibits the growth of different microorganisms [29, 31, 36]. Notwithstanding, no inhibition was observed in cultures in TSA medium in the present work, neither in experiments with hot pepper extracts nor with ethanolic solutions of capsaicin. On the contrary, results were positive with pepper extracts and capsaicin in experiments in liquid medium against *B. subtilis*. In fact, this microorganism was proved to be suscetible to capsaicin [36]. The lack of inhibition in solid medium could be explained by the hydrophobic character of capsaicin, and its difficulty to diffuse through TSA solid medium.

No inhibition of *E. coli* by capsaicin was observed in liquid medium. However cultures with pepper extracts showed a small drop in the optical density, suggesting a perceptible slight inhibition of growth. As pure capsaicin doesn't affect *E. coli*, it might be thought of the presence of other substances with antimicrobial activity in pepper extracts. Mokhtar and colleagues described the antimicrobial activity of pepper polyphenols and substances such as caffeic acid, quercetin and kaempferol against *Staphylococcus aureus*, *Salmonella typhimurium* and *Pseudomonas aeruginosa* [37].

The tolerance of *E. coli* to capsaicin has been reported in other studies. Jones et al reported that capsaicin specifically inhibited the growth of *H. pilori* but did not affect to *E. coli* [29]. Extracts from bell pepper [34] and jalapeño peppers [35] did not affect the growth of E. *coli* either. A feasible reason why capsaicin does not affect to *E.coli*, is that this bacterial species is a commensal microorganism, a consequence of an adaptive process to the human and animals digestive tracts. Thus, the ingestion of hot peppers could have a protective effect against the intake of pathogenic bacteria without altering the own gastrointestinal bacterial flora.

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MY OWN IDEAS

Elena de la Torre Amat

It is the first time that I have participated in a project like this in which one does experiments, goes to advice, writes articles and many other things. This being a new experience has been taken with great enthusiasm, although the coronavirus has not helped us much, it has not made us give up at any time.

What I liked the most about the project has undoubtedly been to see how other people were interested in what we were discovering that they were seeing through the blog, I also found the advice very interesting in which we discovered other projects that were also being done We also saw things that we already knew, such as that science can change many things in our lives. Although it seems that we do not live with science every day, we may not make great discoveries like researchers or experts, but if we can help to do new research on other topics, such as peppers, in which you discover things that you do not you knew they could happen.

For me it has been a pleasure to be able to count on experts when carrying out this project, without their help we would not have been able to get here, although we still have many things to do, many experiments to do and many things to learn.

I am sure that this project has also been a new and fantastic experience for the rest of my colleagues, who despite the covid have been there for the project and have done a great job.

Paula Entrena

At the beginning, when we started the project I thought: how are we going to investigate with peppers? If peppers are that, peppers. What are we going to discover about them?

I started with little desire, I didn' t know if I could devote enough time to the project or to the rest of the subjects ...

But, from the first moment, it absorbed me in such a way that I could not stop working, searching and investigating.

In each of the classes, I was filled with curiosity and desire to continue.

Thanks to this project I have learned the unimaginable about science and I have managed to open my eyes to something that is beginning to make me want to learn more every day: Research/Investigation. And this is why my academic and professional future I am beginning to visualize.

All the work that we have carried out is very important, since it would never have occurred to us to research peppers and the conclusions we came to surprised us from the beginning.

To continue this project, more research could be done on the possible beneficial effects that quercetin in peppers can cause; since this flavonoid together with vitamin C, can prevent the virulence of the Covid-19 pathogen according to some recent studies.

What if we discovered that bell peppers are a natural barrier against coronavirus infection?

Al principio, cuando empezamos el proyecto pensé: ¿cómo vamos a investigar con pimientos? Si los pimientos son eso, pimientos. ¿Qué vamos a descubrir acerca de ellos?

Empecé con pocas ganas, no sabía si podría dedicarle el tiempo suficiente al proyecto ni al resto de asignaturas...

Pero, desde el primer momento, me absorbió de tal manera que no podía parar de trabajar, buscar e investigar.

En cada una de las clases, me iba llenando de curiosidad y ganas por seguir adelante.

Gracias a este proyecto he aprendido lo inimaginable sobre ciencia y he conseguido abrir los ojos hacia algo que me está empezando a producir muchas ganas de aprender cada día más: La investigación. Y es por esto por lo que mi futuro académico y profesional lo estoy empezando a visualizar.

Es muy importante todo el trabajo que hemos llevado a cabo, ya que nunca se nos habría ocurrido investigar sobre pimientos y las conclusiones a las que fuimos llegando nos sorprendieron desde el principio.

Para continuar este proyecto se podría investigar más sobre los posibles efectos beneficiosos que puede ocasionar la quercetina de los pimientos; ya que este flavonoide junto con la vitamina C, pueden impedir la virulencia del patógeno de la Covid-19 según algunos estudios realizados recientemente. ¿Y si descubrieramos que los pimientos son una barrera natural frente a la infección por coronavirus?

Ignacio Mengíbar López

From my point of view, this project taught me to use different kinds of instruments in our lab, to accept a negative result of an experiment and go ahead with curiosity and motivation, and I learn more about work with my schoolmates. This summer I ´m going to investigated about the transmission of capsaicin between different kinds of pepper.

Ainhoa Yolanda Moreno Duperón

In my opinion, this has been an amazing and funny project. How you can learn so impressive things from something so simple as a pepper, from a fruit that grows in a plant, and the knowledge of people about them: if they are sweet or hot, green, yellow, orange or red. Most of the classmates who have participated in this project were enthusiastic for learning the complex things a simple pepper has, as its nutritional value, the capsaicin levels, using automatic pipettes, petri dishes or even microorganisms that affect plants and animals to analyze their effect.

It is a beautiful project, with lot of things to learn. It should be continued, looking for information and investigating more thing about peppers, with the same people or new ones, in order other people know as well about peppers. It has been a wonderful project although things didn’t result as we had expected, but we tried again and again until our results confirmed other experiments. We should continue our research about peppers.

Marta Roldán Martín

The project has made me enjoy a lot and thanks to it I have learned things like working together with my classmates in a laboratory in a professional way, doing experiments that few kids our age can claim to have done and working hand in hand with high level researchers.

Also with the project I have improved the writing of scientific articles, something that is very useful and that in the future will help me, this has been possible from the different participations that we have all made in the blog and that have later served as a model when we prepared our article

In addition, in my case I I have had an important role participating in the recording of the project presentation and answering questions during the congress, what is more exciting, in spite of that I think everyone had an important role during the development of the project and I think that all of my classmates will agree with me in the fact that been part of this has been an important oportunity to see how the scientific world really works.

For all this, I believe that it has been a very enriching experience and that it should not end with the publication of this article. Many questions remain to be answered.

Is there a relationship between capsaicin and vitamin c in samples of other species of peppers? Is there an antimicrobial effect in other species of peppers?

We can also study the degradation of pepper properties over time or the degradation of the properties from extreme conditions such as a very high temperature and also study the effect that its consumption could have on these conditions in humans.

These are some of the many questions that we can ask from this study, so as I said, this project does not end here, let's get down to business and keep asking more questions to answer.

Laura Rodríguez López

This project has been very interesting to me, it has made me fell interested in plants scientifically, a branch of Biology that I love. I think it is important, since these types of research projects let to advance in science, and this is very important. I would like to participate in more researches and experiments, especially if it has to do them with plant biology.

Julia Shan Vida Lara

I have found the project amazing; it is a project that has made me interested in something as simple as a pepper. We started doing it without knowing how it was going to end, and all together, we divided into groups and did experiments to investigate, I was in charge of putting the tables together and drawing conclusions about them together with the others in my group. I would like to know more about other types of pepper and to continue developing this project as well as knowing this same information about another species of pepper, from other places.

My experience at the congress was an amazing experience, and I really enjoyed hearing about different scientific projects, as interesting as they were, and being able to ask questions to each group, helped me to understand them better.