PhD Course in Food Science, 36th cycle

Final activity report

Stay at University of Parma

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During my permanence at the University of Parma, I have dedicated most of the time to work in the Department of Food and Drug, under the supervision of Prof. Daniele Del Rio and Prof. Pedro Mena Parreño. I have also collaborated with the Department of Medicine and Surgery, and carried out some activities in remote (smart-working).

The work carried out at the **Department of Food and Drug** allowed me to learn about *in vitro* colonic fermentation techniques, as well as to develop the experimental part of my thesis related to the colonic metabolism of phytochemical compounds of fresh garlic, black garlic, fresh onion, and black onion. First, I had to learn the experimental protocols, for which I was assisted by another PhD student of the department, who taught and supervised me during this process. This was followed by the *in vitro* colonic fermentation of my samples, previously digested by *in vitro* gastrointestinal digestion, and then freeze-dried.

Faecal fermentations were carried out on a total of 162 samples, collected at 2, 4, 8 and 24 h with their respective controls. Subsequently, optimisation of the extraction of the compounds of interest from the faecal matrices was performed. For this purpose, tests were carried out with different solvents and it was observed with which solvent and under which conditions the best extraction yield was obtained for phenolic and organosulfur compounds. Thus, it was observed that the best extraction solvent for phenolic compounds was ethyl acetate acidified with 0.1% formic acid and for organosulfur compounds it was ethanol acidified with 0.1% formic acid.

Once the best extraction solvent was clarified, the optimisation of the analytical method was carried out. For phenolic compounds, we worked with chromatographic conditions already used for previous studies. However, it was necessary to create up to five chromatographic methods to analyse the complete profile of phenolic compounds and their catabolites by UHPLC-LIT-MS, adapted to each food matrix. The complete analysis of all samples with the methods for the identification and quantification of phenolic compounds required more than 2 weeks of analysis. On the other hand, the chromatographic analysis of the organosulfur compound profile needed to be optimised as it was the first time that this type of compounds was worked with in the laboratory. For this purpose, the few standards commercially available were used to modify the conditions in order to optimise the signal detected by the equipment. Finally, the analysis of the organosulfur compound profile by UHPLC-LIT-MS was also carried out, requiring at least another two full weeks. The files obtained from the UHPLC-LIT-MS analysis were processed by a software called Xcalibur v.2.0, which I was taught to use to carry out the identification and quantification of the different compounds and catabolites detected in the samples. Data processing, statistical analysis and preparation of manuscripts for publication was carried out during the smart-working period with the continuous support of my supervisors and their team.

In the **Department of Medicine and Surgery**, under the supervision of Dr. Massimiliano Bianchi, I was working with ileofecal fluids from subjects who had consumed mango, with the aim of assessing whether mango consumption could be related to any preventive or treatment effect against an inflammatory response. The aim of this collaboration was to learn and train in new cell co-culture techniques. The research group of Prof. Del Rio and Prof. Mena are also involved in this study and discussion was carried together.

The experiments were carried out with a co-culture of Caco-2 and HT29-MTX cells. These cell lines were cultured separately until they reached the necessary cell density and regained their activity, then trans-wells

were seeded, allowing the cells to grow by simulating the intestinal epithelial barrier. This co-culture is maintained for 21 days, changing the apical and basolateral medium every 3 days. We performed different types of experiments on these cell lines. Firstly, we tried to simulate as closely as possible the physiological conditions, for which we evaluated whether high concentrations of ileofecal fluid were toxic for the cells. We also assessed the minimum effective dose of cytokines, and we worked with an extract of ileofecal fluid with a total polyphenol content that mimicked real physiological conditions.

The different experiments carried out with the cells were monitored the transepithelial electrical resistance (TEER) every 24 h. In addition, cell viability was determined by staining with resazurin and then performed a Western-Blot, a technique used to detect specific proteins (in our case, structural proteins of the cell barrier formed by our culture, by their reaction with primary and secondary antibodies). Moreover, we carried out two more experiments in which we added the samples of the subjects to the culture, both before and after consumption of mango, for 24 h, to subsequently add the cytokine mix and observe whether mango consumption had any effect.

Although I did not obtain analytical results directly related to my thesis, this knowledge will serve as a basis for future research in cell lines to evaluate the bioavailability and metabolism of phytochemicals from black garlic and black onion in Caco-2 cell lines as a model of human intestinal absorption, as well as to study the chemopreventive effect of these compounds in Caco-2 and HT29 cell lines.

The work within these two departments of the University of Parma was carried out during my stay from October 10th, 2020 to April 10th, 2021. Then, due to the delay caused by the COVID-19 pandemic in the development of laboratory activities planned for my thesis (the beginning of the 1 year period was scheduled for March 2020), it was decided to continue the research plan of activities related to the University of Parma in remote through smart-working. This allowed me to continue with the research plan of activities related to the University of Cordoba while carrying out also the research plan related to the University of Parma. This decision was done taking into account the research programme of the thesis, the activities to be carried out, and the possibility to conduct these activities online, in agreement with all the Ph.D. tutors.

As for this smart-working period (from April 12th 2021 to October 13th 2021), the data processing of the analyses performed during my stay in the Department of Food and Drug was carried out. This consisted of the identification and quantification of metabolites determined by UHPLC-LIT-MS analysis during the *in vitro* colonic fermentation, in particular (poly)phenols and organosulfur compounds from fresh garlic, black garlic, fresh onion and black onion. Data processing has been carried out remotely, as well as the statistical analysis of the data and the development of the manuscripts derived from the results obtained in this research. In fact, the work carried out in the Department of Food and Drug has resulted in two manuscripts entitled: "*In vitro* colonic fermentation of fresh and black garlic" and "Effect of *in vitro* colonic fermentation on the stability of fresh and black onion bioactives" and sent to journals with a high impact index (Q1 in the area of Food Science & Technology), the Journal of Agricultural and Food Chemistry and Food & Function, respectively.

Additionally, I have also actively collaborated in the FOODPHYT project, specifically in the first objective of this project, which aims to make available consolidated knowledge on metabolism and cardiometabolic health effects of food phytochemicals in an open-access database. In this sense, I have collaborated in covering the data collection of thiosulfinate and flavanone metabolites through systematic searches and data extraction procedures. This will also serve for the preparation of a couple of publications with different international groups participating from this project. This activity, initially not related to my research plan, has been a quite interesting opportunity expanding my knowledge on the metabolism of these compounds and in the techniques used to perform systematic searches.

A timeline of the research activities carried out is provided below:

Month / Activity	oct-20	nov-20	dic-20	jan-21	feb-21	mar-21	apr-21	may-21	jun-21	jul-21	aug-21	sep-21	oct-21
Department of Food and Drug													
In presence period													
Smartworking period													
Collaboration with the Department of Medicine and Surgery													

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Ph.D. Tutor: Prof. Daniele Del Rio, on behalf of all the tutors