Collège Doctoral du Languedoc-Roussillon

THÈSE

Pour obtenir le grade de

Docteur

SupAgro

Ecole doctorale

Sciences des Procédés – Sciences des Aliments

Unité Mixte de recherche (UMR)

Spécialité : **Biotechnologie, microbiologie**

Présentée par

M^A CRISTINA GARCIA MUÑOZ

BIOCONVERSION DES ELLAGITANNINS DE LA MURE TROPICALE DE MONTAGNE (*Rubus Adenotrichos*) ET RELATION AVEC L'ÉCOLOGIE DU MICROBIOME INTESTINAL

Soutenue le 12 Décembre 2013 **devant le jury composé de**

M. Olivier DANGLES, Université d'Avignon, INRA	Professeur	Président du jury
M. Lars Ove, DRAGSTED Université de Copenhagen	Professeur	Rapporteur
Mme Joëlle QUETIN-LECLERCQ, Université Catholique de Louvain	Professeur	Rapporteur
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Montpellier SupAgro Institut des régions chaudes

DEDICATION

I dedicate this thesis to my mother Maria Yolanda Muñoz who taught me the moral values based on honesty, compassion, courage, and forgiveness, to become an integral person in both personal and professional life.

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ABSTRACT

Consumption of dietary ellagitannins (ETs) could be associated mainly with prevention of cardiovascular diseases and regulation of hormone-dependent cancers. Nonetheless, ETs are not bioavailable as such; therefore, after being partially converted into ellagic acid (EA) in the upper gastrointestinal (GI) tract, they undergo sequential bioconversion in the colon by gut microbiota into urolithins, a more bioavailable and bioactive group of molecules that persist up to 4 days at relatively high concentrations in urine. Variability of urolithin excretion in urine is high and three main groups, "no or low urolithin excreters," "predominantly UA derivatives excreters" and "predominantly UB derivatives excreters," were observed on a cohort of 26 healthy volunteers. These categories were also unambiguously observed following the total excretion of main ETs' metabolites over a 4 day period after ingesting one shot of juice, and at different periods of time along one year. Although relatively high inter- and intra-individual variabilities were observed, individuals preserved their status during various intervention periods with different amounts of ETs ingested. UPLC-PDA and ESI-Q-TOF/MS^1 and MS^2 allowed the tentative assignment of an identity to 15 other ETs metabolites in urine, but this profiling did not allow the discrimination of any other compounds aside from UA or UB derivatives. In-vitro fermentation of ETs and EA with fecal stools showed a specific metabolic pathway ending in the production of UA. Nonetheless, metabolites excreted in-vivo are much more complex, highlighting strong interactions between host excretory system and composition of gut microbiota. Hepatic recirculation and additional bioconversion of Phase II metabolites in the colon may explain predominant excretion of UB in some volunteers. Microbiota ecology assessed by PCR-Denaturing Gradient Gel Electrophoresis (DGGE) fingerprint method allowed the association of some microorganism species to higher capacity of bioconversion of dietary ETs into urolithins.

Keywords: Ellagitannins, blackberry, urolithin, colonic metabolites, ETs degradation patterns, gut microbiota, gastrointestinal tract,

RESUME

La consommation d'aliments riches en ellagitannins (ETs) pourrait être associée principalement à la prévention des maladies cardiovasculaires et la régulation des cancers hormono-dépendants. Néanmoins, les ETs ne sont pas biodisponibles en tant que tel et, après avoir été partiellement transformés en acide ellagique (EA) dans le tractus gastro-intestinal (GI) supérieur, ils sont métabolisés dans le côlon par la flore intestinale en urolithines, un groupe de molécules plus biodisponibles et bioactives qui peuvent persister jusqu'à 4 jours à des concentrations relativement élevées dans le plasma et l'urine. La variabilité de l'excrétion des urolithines dans l'urine est importante et à partir d'un échantillon de population de 26 volontaires sains, trois groupes principaux d'individus ont pu être distingués : "faible ou non-excréteur d'urolithin », « Excréteur prédominant d'UA et dérivés» et « Excréteur prédominant d'UB et dérivés»". Ces groupes ont également été observés en considérant la cinétique totale d'excrétion sur une période de 4 jours après ingestion du jus et à des périodes différentes tout au long d'une année. Bien que les variabilités inter-et intra-individuelles soient relativement élevées, les individus conservent leur statut au cours des différentes périodes d'intervention même en modifiant les quantités d'ETs ingérées. L'analyse par UPLC-PDA/ESI-Q-TOF/MS^2 a permis d'attribuer hypothétiquement une identité à 15 autres métabolites d'ETs dans l'urine, mais le profilage métabolomique n'a pas permis de discriminer d'autres composés exceptés les dérivés d'UA ou d'UB. La fermentation in-vitro des ETs et EA, par les matières fécales a montré une voie métabolique spécifique qui débouche sur la production d'UA. Néanmoins, les métabolites excrétés *in vivo* sont beaucoup plus complexes ce qui met en évidence de fortes interactions entre le système excréteur de l'hôte et la composition du microbiote intestinal. La recirculation hépatique suivie par une re-conversion des métabolites de phase II dans le côlon permettrait d'expliquer l'excrétion d'UB chez certains volontaires. L'écologie spécifique de la flore intestinale évaluée par la méthode des empreintes PCR-DGGE a permis d'identifier quelques microorganismes associés à une plus grande capacité de bioconversion des ETs en urolithins

TABLE OF CONTENTS

DEDICATION 3

ACKNOWLEDGEMENTS 4

ABSTRACT 7

RESUME 8

LIST OF PUBLICATIONS 12

LIST OF TABLES 13

LIST OF FIGURES 15

LIST OF ABBREVIATIONS 19

1 INTRODUCTION 21

2 LITERATURE REVIEW 24

2.1 ELLAGITANNINS 24

2.1.1 STRUCTURE 24

2.1.2 OCCURRENCE 25

2.1.3 ELLAGITANNINS BIOAVAILABILITY 27

2.1.4 POTENTIAL IMPACT ON HEALTH 31

2.1.5 TOXICITY OF DIETARY ELLAGITANNINS 38

2.2 GUT MICROBIOTA AND BIOCONVERSION OF DIETARY COMPOUNDS 38

3 MATERIALS AND METHODS 44

3.1 REAGENTS AND MATERIALS 44

3.1.1 CHEMICALS 44

3.1.2 GROWTH MEDIUM 44

3.1.3 BLACKBERRY JUICE, BBJ 44
3.2 ANALYTICAL METHODS 45
3.2.1 SAMPLE PREPARATION 45
3.2.2 ANALYSIS PERFORMED FOR THE IDENTIFICATION AND QUANTIFICATION OF ETs AND THEIR METABOLITES 46
3.2.3 CLINICAL STUDIES WITH HEALTHY VOLUNTEERS 60
3.2.4 IN-VITRO FERMENTATION WITH STOOLS 62
3.2.5 PROFILING OF INTESTINAL MICROBIOTA 65
3.2.6 MULTIVARIATE ANALYSIS OF DGGE PROFILES 76
4 METABOLIC FATE OF ELLAGITANNIN'S COLONIC DERIVATIVES 79
4.1 Identification and quantification of Ellagitannins in blackberry juice 79
4.2 IDENTIFICATION OR ANNOTATION OF ETs METABOLITES PRESENT IN URINE AFTER INGESTION OF BBJ 79
4.2.1 IDENTIFICATION OF UAG AND UBG IN URINES 80
4.2.2 ANNOTATION OF OTHER ETS METABOLITES FOUND IN URINE 82
4.3 DIVERSITY OF URINARY EXCRETION PATTERNS IN HUMANS 85
4.3.1 INTER-INDIVIDUAL VARIABILITY OF URINARY EXCRETION OF UROLITHIN OVER A 4-DAY PERIOD 86
4.3.2 KINETIC EXCRETION OF UROLITHIN A AND UROLITHIN B 89
4.3.3 INTRA-INDIVIDUAL VARIABILITY OF URINARY EXCRETION OF UROLITHINS 92
4.4 IN-VITRO SIMULATION OF COLONIC FERMENTATION 94
4.4.1 IDENTIFICATION OF ETs METABOLITES PRESENT IN FERMENTATION BROTH 94
4.5 VARIABILITY OF STOOLS' ABILITY TO PRODUCE ETs METABOLITES 98

4.5.1 EFFECT OF SUBSTRATE IN THE PRODUCTION OF UROLITHINS 99

4.5.2 SUGGESTED BIOCONVERSION PATHWAY OF ETs TO UROLITHIN IN-VITRO 101

4.6 POTENTIAL INTERACTION BETWEEN MICROBIOTA AND HOST 102

5 MICROBIOTA ECOLOGY AND ABILITY TO PRODUCE UROLITHINS 105

5.1 RESEARCH OF CORRELATION BETWEEN GUT ECOLOGY AND IN-VIVO ABILITY TO EXCRETE UROLITHINS 105

5.1.1 PCR-DGGE PROFILING OF THE 26 VOLUNTEERS 105

5.1.2 RELATION WITH UROLITHIN EXCRETERS STATUS DETERMINED ON A URINE SPOT 109

5.2 RELATION BETWEEN MICROBIOTA AND *IN-VITRO* BIOCONVERSION OF UROLITHIN 115

5.3 IDENTIFICATION OF FECAL BACTERIA INVOLVED IN UROLITHIN METABOLISM 118

5.4 EFFECTS OF ETs INGESTION ON MICROFLORA PROFILE 120

6 CONCLUSIONS 124

7 BIBLIOGRAPHY 128

LIST OF PUBLICATIONS

Results from this study have been published or will be published like scientific papers:

Garcia-Muñoz, Cristina and Vaillant Fabrice. Metabolic Fate of Ellagitannins: Implications for Health, and Research Perspectives for Innovative Functional Foods. Critical Reviews in Food Science and Nutrition

Garcia-Muñoz, Cristina; Hernandez Lorena, Pérez, Ana M, Vaillant Fabrice. Diversity of urinary excretion patterns of primary ellagitannin colonic metabolites after ingestion of tropical highland blackberry (*Rubus adenotrichos*) juice, submitted and accepted by Food Research International.

Garcia-Muñoz, Cristina, Meile Jean Christophe, Hernandez, Lorena, Vaillant, Fabrice Microbiota ecology affects urolithin production during in-vitro simulation of colonic fermentation of blackberry juice**.**

Garcia-Muñoz, Cristina; Hernandez Lorena, Pérez, Ana M., Vaillant Fabrice Colonic microflora and the bioconversion of ellagitannins from tropical highland blackberries in to bioactive molecules. International Conference on Nutrigenomics - INCON 2012 *"Gene-Diet Interaction for Personalized Health and Disease Prevention*" San José, Costa Rica. October, 1fst to 4th, 2012

Garcia-Muñoz, Cristina; Hernandez Lorena, Mertz, Christian, Vaillant Fabrice Approaching to metabolic pathway of urolithin production from blackberry juice. International Conference on Nutrigenomics - INCON 2012 "Gene-Diet Interaction for Personalized Health and Disease Prevention" San José, Costa Rica. October, 1fst to 4th, 2012.

LIST OF TABLES

Table 2.1 Major ellagitannins food sources....26
Table 2.2 Content of main phenolic compounds in blackberries (*R. adenotrichos*) at three maturity stages. [31] 27
Table 2.3 Contents of main phenolic compounds in blackberries (*R. adenotrichos*) at three maturity stages.[35] 28
Table 2.4 Recent prospective clinical studies of consumption of food rich in ellagitannins33
Table 2.5 Summary of intervention studies with animals to evidence health benefits of dietary ETs....34
Table 2.6 Summary of some recent results obtained on cell-models by ETs, EA and urolithins....35
Table 2.7 Main studies on bioconversion of dietary compounds into bioactive colonic metabolites [130] 41
Table 3.1 Relative efficiency of electrospray ionization of common chemical functional groups (Fiehn Laboratory, UC Davis)....53
Table 3.2 PCR primers targeted for 16S rRNA gene, without GCClamp [197] 67
Table 3.3 Encoding for the four bases and for ambiguous positions in DNA sequences 75
Table 4.1 Contents of ellagitannins in blackberry (*Rubus adenotrichos*) juice....79
Table 4.2 UPLC-DAD/ESI(+)-Q-TOF/MS and MS2 analyses of ellagitannins colonic metabolites found in urines of volunteers 83
Table 4.3 UPLC /ESI(+)-Q-TOF/MS profiling of the main ETs metabolites excreted in urine during the 4 days after ingestion of blackberry juice for nine individuals (letters) and for different intervention periods (numbered in chronological order) 89
Table 4.4 Total excretion of urolithins UA and UB and percentage of conversion in relation to ingested ETs 91
Table 4.5 Chromatographic and mass spectra characteristic of ellagitannins metabolites found in broth culture incubation of EA and BBJ inoculated with fecal stools from urolithin excreters....95

Table 4.6 Main ETs metabolites released along the degradation of ETs.a) EA, b) UM5, c) Iso UD1, d) Iso UD2, e) Iso UD3. 96
Table 4.7 Main ETs metabolites released along the degradation of ETs. a)Iso UC1, b) Iso UC2, c) Iso UC3 d) UA 97
Table 5.1 Concentration of urolithin A and B derivatives in a urine spot collected at 51±5 h, after ingestion of one shot of 250 ml of blackberry juice. 109
Table 5.2 EA and UR Index of stools from 9 individuals 116
Table 5.3 Recapitulation of retardation factors (Rf) of the bands found as discriminative in the different trials 118
Table 5.4 Results of the sequence similarities of the excised bacterial bands from different DGGE gels 119

LIST OF FIGURES

Figure 2.1: A. Puncalagin; B. Lambertianin C10, and the HHDP group. [3] 25
Figure 2.2 Phylogenetic tree of the microbial diversity in the human gastrointestinal tract, based on the 16S rDNA bacterial sequence data [2] 39
Figure 3.1 HPLC-MS and B) UPLC-MS analysis of mouse urine in a 3-dimensional map to observe the differences on the information generated [1] 47
Figure 3.2 Comparing speed, sensitivity and resolution in HPLC and UPLC analysis [2, 3] 47
Figure 3.3 Calibration curve of EA, for quantification of ellagitannins by HPLC, following the method proposed by Mertz, 2007. 50
Figure 3.4 UV chromatogram of blackberry (*Rubus adenotrichos*) juice. 50
Figure 3.5 Calibration curve of EA, UA and UB for quantification of ellagitannins in BBJ, and ETs metabolites in urine and broth culture incubation. 51
Figure 3.6 Basic information provided by UPLC: UV chromatogram-Area under the curve and the UV spectra for m/z+ 213.0552 ion is illustrated 51
Figure 3.7 Electrospray ionization principles[190] 53
Figure 3.8 Illustration of a mass spectrometer MS V-Mode and each one of their parts, along with UPLC that become a powerful tool for metabolite analysis 56
Figure 3.9 Basic information obtained from MS: A. Total ion chromatogram (TIC) B. extracted ion chromatogram m/z and C. Exact mass measured spectrum and elemental composition report 58
Figure 3.10 Scheme of the clinical test followed to approach ETs metabolism. 61
Figure 3.11 Layout of study design, different steps followed 62
Figure 3.12 Scheme of *in vitro* test followed to identify microbial metabolites and potential microbial species involved in ETs degradation 64
Figure 3.13 PCR process. 1. Denaturing, 2. Annealing, 3. Extension, 4. Cycles [202] 66
Figure 3.14 DGGE principles: voltage, gradient, and direction of moving 68
Figure 3.15 Spatial distribution of microbes at a selected site in the gastrointestinal tract [1] 69

Figure 3.16 Example of the calibration of the retardation factor (Rf) between lanes and two different gels 73
Figure 3.17 Process followed by Phoretix 1D® to obtain aligned bands between lanes and gels and Excel file with band % corresponding to each retardation time 74
Figure 3.18 Example of DNA sequences provided by GATC BIOTECH 76
Figure 4.1 Example of UV chromatogram (at 278 nm) of a urine sample from a volunteer collected 3 days after ingestion of blackberry juice, including peaks and UV spectra of glucuronide forms of urolithin A (UAG) and B (UBG). 80
Figure 4.2 MS^2 Fragmentation of urolithin A and urolithin B 81
Figure 4.3 MS^2 fragmentation of urolithin A glucuronide 81
Figure 4.4 Example of MS2 fragmentation pattern of Ellagic Acid, urolithins: UM5, UD UC, dimethyl elagic acid glucuronide (DMEAG), methyl urolithin A (MUA) and dimethyl-urolithin C (DMUC), and tentative prediction of some fragment ions 84
Figure 4.5 Concentration of primary urolithin A and B derivatives (aglycone and glucuronides forms) in a urine spot, from 26 volunteers, collected at 51±5 h after ingestion of 250 mL of blackberry juice diluted (50:50) with water. 85
Figure 4.6 Total conversion of ETs to UA and UB derivatives (aglycone and glucuronide determined by UPLC/DAD) excreted in urine over 4 days after ingestion of 250-mL of tropical blackberry juice for 9 individuals (C,D,F,G,H,I,O,R,W) at different interventions. 87
Figure 4.7 Urolithin UA and UB detected in every one of the samples of urine excreted for 5 individuals up to seven days after they ingested a 250-ml of BBJ. 90
Figure 4.8 Excretion kinetics of UA(on the right) and UB(on the left) derivatives for four individuals (C,G,H, and I numbered in chronological order) performed at different periods of time around one year 93
Figure 4.9 Example of UV chromatogram (at 278 nm) from a fermentation broth (EA as substrate, 120 h fermentation, stool 96 h inoculate from individual G 95
Figure 4.10 Primary ellagitannins metabolites assessed after 24, 72 and 120 h of batch anaerobic incubations of blackberry juice (A) and ellagic acid (B) with fecal stools donated by nine volunteers (A, B,C,D,E,F,G,H,I), previously qualified as urinary 99

Figure 4.11 Suggested metabolite pathway occurring during incubation of pure ellagic acid with active fecal stool from urolithin excreter..... 102
Figure 4.12 Schematic representation of proposed metabolic pathways of the excretion of the main metabolites identified in human urine after ingestion of tropical highland blackberry juice 104
Figure 5.1 Hierarchical cluster analysis of the 26 PCR-DGGE profile PCR-DGGE fingerprint corresponding to amplicons of the V3 region of 16S rDNA of faecal bacteria from stool (Letter and number of lane corresponds respectively to volunteers identity and number of repetition; Ctrl is a control lane with *lactobacillus plantarum* and *E.Coli*)..... 107
Figure 5.2 Complete linkage cluster analyses of 1-r (1 minus the Pearson coefficient between lanes) (Letter and number of lane corresponds respectively to volunteers identity and number of repetition; Ctrl is a control lane with *L. plantarum* and *E.Coli*) 108
Figure 5.3 Principal Component Analysis (PCA) of PCR-DGGE profile for 26 volunteers and "urolithin excreters status" (N for "No urolithin excreters", UA for "Predominant UA excreters" and UB for "Predominant UB excreters". 110
Figure 5.4 Score scatter plot of 26 individuals (plus some repetition) for PLS-DA regression of PCR-DGGE bands "X" taking as response ("Y") the urolithin excreters status (R2Xcum=0.146, R2Ycum=0.68, Q2cum=0.49, CV-ANOVA<0.05). 111
Figure 5.5: Loading plot of the different bands from PCR-DGGE analysis "X" for PLS-DA regression of PCR-DGGE bands "X" taking as response ("Y") the urolithin excreters status (R2Xcum=0.146, R2Ycum=0.68, Q2cum=0.49, CV-ANOVA p-Value =0.003) (Size of "X" variable..... 112
Figure 5.6 PLS-DA regression coefficients with confidence intervals for the three different groups, for each bands of the PCR-DGGE..... 113
Figure 5.7 PLS variable importance for projection for each bands of the PCR-DGGE with confidence intervals (R2Xcum=0.0525, R2Ycum=0.6, Q2cum=0.39, CV-ANOVA for UA P-value =0.12, for UB 0.038 and for total urolithin 0.048)..... 114
Figure 5.8 PLS regression coefficients for the "UB predominant excreters" group. 115
Figure 5.9 Scatter plot (A) and Loading plot (B) of PCR-DGGE bands "X" (I) and regression coefficients with confidence interval (C) obtained by OPLS taking as response ("Y"), bioconversion ability index of ellagitannins into ellagic acid (EA index) (C1) and

into urolithin (UR index) (C2) (R^2X_{cum}=0.232, R^2Y_{cum}=0.93, Q^2_{cum}=0.60, CV-ANOVA p-value for EA index and UR index P-value 0.07 and 0.04 respectively) (In graph I, size of loading plot is proportional to "X" variable importance for projection of the model)..... 117
Figure 5.10 Percentage of molar conversion of EA or ETs (EA equ.) into primary ET-metabolites, when blackberry or ellagic acid (EA) are fermented 120 h with stools from one individual (individual G) collected at 48h and 96h after consumption of 250 ml of blackberry 122
Figure 5.11 DGGE gel of PCR products of variable V3 region from 16S rDNA of fecal samples from 3 individuals (I , F and C) that donated fecal stool samples at 0, 48, 96 or 120 hours after ingesting one shot of 250-ml of BBJ..... 123

LIST OF ABBREVIATIONS

BBJ:	Blackberry juice
BEH.	Ethylene bridged hybrid
DAD:	Diode Array Detector
DGGE:	Denaturing Gel Gradient Electrophoresis
DMEA:	Dimethyl ellagic acid
DMSO:	Dimethyl sulfoxide
DMUC:	Dimethyl Urolithin C
EA:	Ellagic Acid
EAME:	Ellagic acid Methyl Ether
ELSD	Evaporative light scattering detector (ELSD),
ESI.	Electro Spray Ionization
ET:	Ellagitannins
HPLC	High pressure liquid chromatography
kPa	Kilo Pascal
LC	Liquid Chromatography
LCMS	Liquid Chromatography Mass spectrometry
M.A.S.L	Meters above sea level
MeOH	Methanol
MS:	Mass Spectrometer
MUA:	Methyl Urolithin A
PCA:	Principal Component Analysis
PCR:	Polymerase chain reaction
PDA.	Photo Diode Array
PLS DA.	Principal Least Square Discriminant Analysis
Q-TOF.	Quadrupole Time Of Flight
Si-OH	Silanols group
SPE	Solid phase extraction
SPEC	Solid phase extraction chromatography
UA:	Urolithin A

UAG: Urolithin A Glucuronide
UAS: Urolithin A Sulfate
UB: Urolithin B
UBG: Urolithin B Glucuronide
UBS: Urolithin B sulfate
UC: Urolithin C
UCG: Urolithin C Glucuronide
UCM: Urolithin C methyl ether
UD: Urolithin D
UDM: Methyl urolithin D
UM5: Urolithin M5
UPLC : Ultra Performance Liquid Chromatography

1 INTRODUCTION

Fruits, vegetables, and nuts are important components of a healthy diet, as they are rich sources of vitamins, minerals, dietary fiber, and many other phytochemicals that are important to prevent diseases and ensure health. Numerous epidemiological studies have shown that regular consumption of these food groups help prevent major chronic degenerative diseases such as cardiovascular diseases and certain types of cancer [4]. Most of them show biological activity, following different mechanisms of action such as antioxidant activity, modulation of detoxification enzymes, stimulation of the immune system, reduction of platelet aggregation, alteration of cholesterol metabolism, modulation of steroid hormone concentrations and hormone metabolism, blood pressure reduction, and antibacterial and antiviral activity [5]. According to the World Health Organization (WHO) [6], about 14% of gastrointestinal cancer deaths, 11% of ischemic heart deaths, and 9% of stroke deaths are caused by low intake of fruits and vegetables. Ellagitannins (ETs), a type of plant polyphenol found in berries, pomegranates, pecans, walnuts, wood-aged wine and derived processed foodstuffs [7-13] could be an active protagonist in some chronic disease prevention and in their nutritional management. Nonetheless, this class of polyphenols with very high antioxidant capacity in-vitro has been largely neglected, from the nutritional point of view due to their large and complex chemical structure, and their ability to bind protein that limits their bioavailability. However, they have recently attracted particular interest among scientists because of the high potential of biological activity of their colonic derivatives. Actually, as ETs are not absorbed as such, they could be partially hydrolyzed into ellagic acid (EA), either in the stomach or in the jejunum; and both ETs and EA can reach the colon where they can also exert local benefits within the gastrointestinal tract [14]. However ETs and ellagic acid can also be used as substrates by gut bacteria and be converted into smaller and more bioavailable compounds called urolithins [15, 16] that have important bioactivity and are able to exert systemic effects, given the time they remain in the circulation system and the possibility to reach target organs and tissues. It has been

stated that urolithins may exert chemopreventive, anti inflammatory effects on the gastrointestinal tract, prostate, and breast cancer and they can also prevent cardiovascular diseases. However, variability in their ability to transform ellagitannins into urolithins has been found in human beings. This has led to classify some volunteers as high or low urolithin producers [17]. Accordingly, some consumers may not be able to obtain all the health benefits from the consumption ETs rich food as the urolithins may not reach sufficient physiological concentrations. To date, there are still many gaps to understand the metabolic fate of ellagitannins. Studies about the inter and intra- individual variability of urolithin production are scarce. The role of microbiota is not completely elucidated and even less the interaction between host and colonic bacteria. The current knowledge on gut bacteria and their role in metabolic pathways involved in polyphenol bio-conversions is still limited. Today, the identification of bacteria species responsible of the transformation of ellagitannins into urolithins is still unknown.

The aim of this study is to tackle these main challenges and, with the help of a multidisciplinary approach, unravel the metabolic fate of ETs and the interaction with gut microbiota.

First we are going to evidence the inter- and intra-variability of urolithin excretion pattern on a cohort of 26 healthy volunteers. This research includes the identification, and in some cases the annotation, of different urolithin compounds excreted in urine. Diversity of excretion pattern will be studied, not only on urine spot, but also for longer periods of up to 7 days after blackberry juice ingestion. Afterwards, the bio-conversion of ETs into urolithin will be simulated in-vitro to try to evidence the ability of gut microbiota to perform this task. From the analysis of these results, we will suggest a bioconversion pathway and discuss the possible interaction between microbiota and the host to explain the excretion patterns observed. In the second chapter, we will try to evidence how gut microbiota composition could influence the ability of the host to produce urolithins. With the help of recently developed culture-independent techniques, we will profile the intestinal microbiota of the volunteers to find common patterns according to the ability to produce urolithin, both in-vivo and in-vitro. This method will create for the first time a tentative identification of the species involved in the bioconversion of ellagitannins.

To achieve these objectives, the study required the articulation of different analytical tools, from different scientific areas, such as biochemistry, microbiology, molecular biology, nutrition and multivariate statistics. Multiple experimental approaches were also tested, with in-vivo studies and in-vitro simulation. Additionally, this research was held in different geographic sites, within the framework of a strong collaboration between the Colombian Corporation of Agricultural Research, CORPOICA, the National Center for Food Science and Technology, CITA, the Center for Natural Products Research, CIPRONA from University of Costa Rica and the International Centre in Agricultural Research for Development (CIRAD) in France. Tropical highland blackberry is a very important local crop in mountain areas with depressions, in Latin-America from south Mexico to the Andean "altiplano", where large communities depend on this crop for survival [18]. This work pays a tribute to these isolated and quiet peasant families that grow a food product that could contribute to a healthier diet for the stressed urban population in developed and developing countries.

2 LITERATURE REVIEW

2.1 ELLAGITANNINS

Plant polyphenols (PP), are secondary metabolites found in fruits, vegetables and derived products composed of aromatic rings with hydroxyl groups bonded. They have several functions in the prevention of degenerative diseases, and are one of the major antioxidants in our diet. [19]. PP are divided in three groups: hydrolysable tannins, condensed tannins, and floratannins, the smallest group [20]. Tannins in higher plants are classified as hydrolysable or condensed. The former are readily hydrolyzed by acids, bases, hot water, or certain enzymes (tannase) [7]. Hydrolysable tannins are divided in two subgroups, gallotannins and ellagitannins, which form the largest group of tannins. Both of them are synthesized from galloyl glucoses, and pentagalloyl glucose [21]. Different aspects of ellagitannins have been studied, such as synthesis, chemical composition and structure, bioavailability, metabolic pathways and their effects on health.

2.1.1 STRUCTURE

Ellagitannins, ET contain two or more neighboring galloyl groups that by oxidation form hexahydroxydiphenoyl (HHDP) units [22]. These HHDP groups are esterified to a sugar, usually glucose (Lei et al., 2001; Lee et al., 2005), and form the largest group of tannins due to diverse possibilities of bonding HHDP residues with the glucose moiety, and their ability to form dimeric and oligomeric derivatives [7]. Depending on the food source ellagitannins exhibit important structural diversity. Punicalagins ($C_{48}H_{28}O_{30}$) and punicalins ($C_{34}H_{22}O_{22}$) are the main ETs found in pomegranates [23] and are the most studied ETs so far. Sanguiin H6 ($C_{82}H_{54}O_{52}$), sanguiin H10 ($C_{68}H_{48}O_{44}$), and lambertianin C ($C_{123}H_{80}O_{78}$) are the major ETs found in berries [7, 8, 24, 25]. Pedunculagin ($C_{34}H_{24}O_{22}$) is the major ellagitanin found in walnuts, while the isomers vescalagin ($C_{41}H_{26}O_{26}$) and castalagin ($C_{41}H_{26}O_{26}$) predominate in oak-aged wine [26, 27]. According to the glucose core, ETs can

be classified into acyclic and cyclic glucose. Vescalagin, castalagin, castavaloninic acid are examples of the first group, while casuarictin, pedunculagin, lambertianin C, Sanguiin H6 are representative ETs of cyclic glucose group [28]. **Figure 2.1**

Whatever the structural complexity of ETs, they all share a common core—the HHDP—even if the number of monomer residues varies according to ET structure. Furthermore, ellagic acid (EA) and direct derivatives are also found in free form in most ET-rich food products [12, 29, 30], especially berries and nuts [7]. When ETs are exposed to acids or strong bases, ester bonds are hydrolyzed and the HHDP acid spontaneously rearranged into the water-insoluble dilactone, EA [7], as a consequence, the units of ellagic acid that can be released per mol of each ET, can be estimated. For instance, each mol of punicalagins **Figure 2,1 A**, has only one HHDP group and then releases only one mol EA. Sanguiin H6 that comprises four HHDP groups, renders four mol of EA; while Lambertianin C, **Figure 2.1 B** that contains six HHDP groups for each mol, can liberates six EA-mol**.**

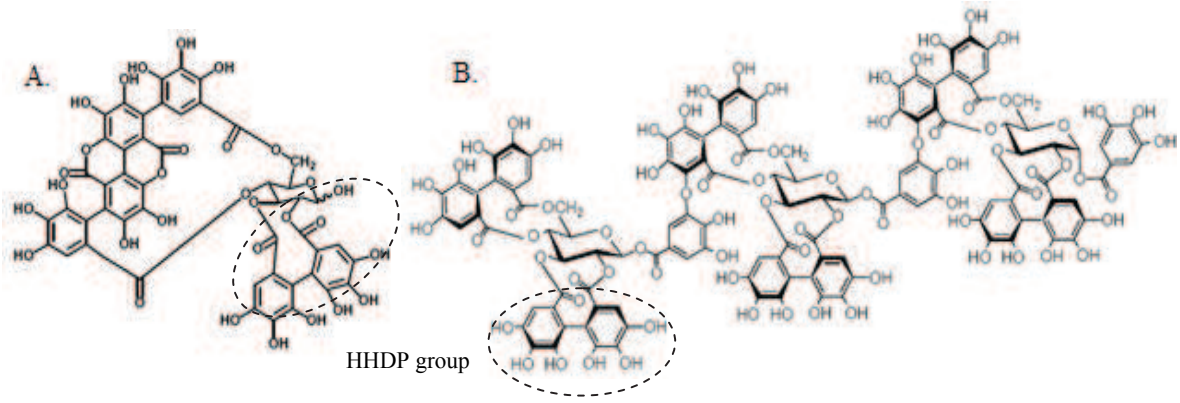


Figure 2.1: A. Puncalagin; B. Lambertianin C10, and the HHDP group. [3]

2.1.2 OCCURRENCE

The content of ETs in plants is expressed usually as EA equivalents, which include ETs such as, free EA, and EA derivatives which can be found in free form in most ET-rich food products [12, 29, 30]. Among the major sources of ETs are trees and fruits, such as oak (*Quercus* sp.) sweetgum (*Liquidambar styraciXua*), linden (*Tilia* sp.), aile (*Alnus* sp.), eucalyptus species, chestnut (*Castanea dentata*), pomegranate, guava, strawberry,

raspberry, blackberry, pistachio, mango, hazelnut, walnut, pepper, plum, apricot, peach, black raisin, red raisin, currant, tea, grape, wines, and aged brandies in oak casks [31-33] but the most important dietary ellagitannins sources are berries and pomegranate, **Table 2.1.** [34]

Table 2.1 Major ellagitannins food sources

Source	**Total ellagitannins**
Pomegranate juice	1500-1900 mg/L
Raspberry	2630 – 3300 mg /kg fresh weight
Raspberry jam	760 mg/kg fresh weight
Strawberry	770-850 mg/kg fresh weight
Strawberry jam	240 mg/1 kg fresh weight
Cloudberry	3150 mg/kg fresh weight
Blackberry	1500-2000 mg/kg fresh weight
Muscadine grape juice	8 – 84 mg/L
Muscadine grape wine	2 – 65 mg/L
Oak aged red wine	9.4 mg/L
Whiskey	1-2 mg / L
Cognac	31-55 mg/L

BERRIES

From the botanical point of view berry is a fruit produced from a single ovary, such as grapes and blueberries, however commercially some aggregate fruits such as blackberries and raspberries are consider as berries and they are amply known and consumed fruits, in fresh and processed forms, such as jams and yogurts [7]. They are also known for their high amount of polyphenols, [35, 36]. However ETs content is affected by internal factors such as variety and ripeness, and external factors, especially those related to climate (temperature, rain), cultural practice (molding, mulching, irrigation, fertilizing), among others. [7, 32, 35-39].

Rubus adenotrichos and Rubus glaucus varieties grown in the tropical highlands of Central and South America and their production have increased to 25% of world blackberry production in 2005 [18, 36]. These varieties are characterized by a higher acidity, whereby they are used mainly by juice industries for blends [18, 36]. Even though casuaricitin, potentillin, pedunculagin and lambertianin D and ET tetramer have been identified in some varieties of blackberries [7], the main phenolic compounds identified in these two blackberry varieties were ellagitannins (Sanguiin H6 and Lambertianin C) and anthocyanins as depicted in **Table 2.2,** while **Table 2.3** shows the effect of ripeness in polyphenol content [32].

Table 2.2 Contents of main phenolic compounds in *Rubus adenotrichos (R.A)* and *Rubus glaucus (R.G)* in mg per 100 g of dry matter[32]

Compound	*R.A*	*R. G*
Anthocyanins		
Cyanidine-3-glucoside	680±20	380
Cyanidine-3-rutinoside	nd	630±20
Cyanidine-3-malonyl glucoside	40±3	nd
Ellagitannins		
Lambertiannin C	598±20	520±30
Sanguiin H-6	420±17	2450±100
Ellagic acid free and conjugated		
Ellagic Acid pentosides	13,5±0,2	43,8±0,2
Ellagic Acid	2,0±0,1	n.d
Ellagic Acid glucoside	8,4±0,3	33,4±0,1
Methyl Pentoside Ellagic Acid	7,5±0,2	2,0±0,1
Ellagic Acid derivatives	n.d	4,3±0,3

2.1.3 ELLAGITANNINS BIOAVAILABILITY

Bioavailability could be defined as the fraction of an ingested nutrient or compound that reaches the systemic circulation, and the specific sites or target tissues where it can exert its biological action; it involves dissolution and absorption, distribution to and disposition in target tissues, metabolism and excretion. [15, 40]

Table 2.3 Contents of main phenolic compounds by HPLC in blackberries (*R. adenotrichos*) at different maturity stages [36].

	Grade 1	**Grade 2**	**Grade 3**
Ellagitannins (mg EA equivalents /g)			
Lambertianin C	12.0 ± 1.0	11.0 ± 1.0	8.0 ± 0.4
Sanguiin H-6	10.0 ± 0.5	9.0 ± 0.4	6.6 ± 0.3
Anthocyanins (mg Cy-3-glc equivalents /g)			
Cyanidin 3-glucoside	1.03 ± 0.03	3.10 ± 0.20	8.30 ± 0.30
Cyanidin 3-(6'malonyl) glucoside	0.111 ± 0.002	0.391 ± 0.005	0.660 ± 0.040
Flavonols (mg quercetin equivalents /g)			
	0.298 ± 0.002	0.231 ± 0.004	0.137 ± 0.002
Ellagic acid derivatives (mg EA equivalents /g)			
	0.300 ± 0.003	0.216 ± 0.004	0.202 ± 0.004
Total (mmol /g)	77.0 ± 5.0	74.0 ± 2.0	69.5 ± 0.3

There are many factors affecting the bioavailability of dietary polyphenols in humans: external factors such as environmental aspects, food processing related factors, (i.e thermal treatments; homogenization), food matrix factors, interaction with other compounds such as proteins, chemical structure of polyphenols, concentration, and host factors like intestinal transit or colonic microbiota [41]. Increasing evidence proposes that gut microbiota is one of the most important factors contributing to the host response towards nutrients [42].

The large and complex chemical structure characteristic of ETs limits their bioavailability, [43-48]. ETs have never been reported in the human systemic circulation system or in urine, even after consumption of high amounts of dietary ETs [14, 24, 46, 47]. Ellagitannins have been detected in plasma in only one case study of laboratory rats subjected to a prolonged diet of ET-containing foods [49]. Despite dietary ETs are not bioavailable, they have been found in relatively low concentrations in the gastrointestinal (GI) tract, including feces [17, 50, 51]**.** In human subjects who have undergone ileostomy, only 23% of the ellagitannin sanguiin H6, ingested by consuming raspberries reaches the ileum (final section of the small intestine), whereas recovery of free EA in ileum fluids increased 2.5 times [52]. This has demonstrated that ETs are partially degraded into EA before reaching this region of the GI

tract. Many studies [14, 43, 44, 49, 50, 52-54] have shown that ETs are partially hydrolyzed in the upper GI tract to EA. However EA, is poorly absorbed due to its low water solubility, its tendency to form insoluble complexes with calcium and magnesium, and to bind to intestinal epithelium [31, 47, 55]. In addition, in the colon and in fecal matter, ETs and EA are found only at relatively low concentrations, which suggest they can be degraded by intestinal microbials during intestinal transit [48, 49, 52, 56].

Other studies show ETs can be hydrolyzed by brush border membrane-bound β−glucosidases, by gut bacterial β-glucosidases in the lower small intestine and colon, or by the physiological conditions of the small intestine (mild alkaline pH, 7.0–7.3) to release free EA, which is absorbed and further delactonized. EA is absorbed and rapidly methyl conjugated by the action of the enzyme COMT (catechol O-methyl transferase) producing EA monomethyl ether and dimethyl ethers. In a second step, conjugation with glucuronic acid yielded the corresponding dimethyl-EA glucuronide which was the most abundant metabolite detected *in vitro* test. [53, 57]. On the other hand, residual ellagitannins that reach the colon are metabolized first into EA by microbial enzymes—tannin-hydrolase and lactonase—which, respectively, cleave the galloyl-glucose residue from the HHDP group and induce enzyme-catalyzed lactonization. Afterwards, farther down the GI tract, other microbial derivatives—the polyhydroxylated dibenzopyranones—are synthesized from EA. Microbial enzymes catalyze the opening of the lactone ring of EA and progressive dehydroxylation to yield urolithins. Dehydroxylation of the polyhydroxylated dibenzopyranones by microbial hydrolases first produces urolithin D (3,4,8,9-tetrahydroxy-dibenzopyranone), then, urolithin C (3,8,9-trihydroxy-dibenzopyranone), urolithin A (3,8-dihydroxy-dibenzopyranone) and finally, the smallest, the more lipophilic and more available dibenzopyranone metabolite urolithin B (3-monohydroxy-dibenzopyranone), is released [9]. Other microbial derivatives may also be metabolized such as 3,8,9,10-tetrahydroxy-dibenzopyranone, isourolithins, and methyl derivatives.

The extent of hydroxyl removal from urolithins and the variety of metabolites produced depend on the time of exposure to gut microbiota; the composition of microbiota; and, eventually, the type of dehydroxylase enzyme involved [58]. The progressive dehydroxylation from penta- to mono-hydroxy-dibenzopyranone are linked to an increase

in lipophilicity and adsorption ability [9, 59]. These metabolites are absorbed passively through the epithelium where they can be conjugated in gut epithelium or undergo a phase II metabolism (methylation, glucuronidation and sulphation) in the liver to render metabolites more soluble that may to facilitate their excretion by urine or secretion in bile. Glucuronidation utilizes a glucuronil group, a very common metabolite supplied by glucose contain in foods, for which urolithins are preferably excreted through conjugation with glucuronic acid under action of UGTs UDP-glucuronosyltransferases. Glucuronides are often eliminated in the bile, to undergo enterohepatic circulation remaining more time in the body, up to 56 hours, which increases the possibility to exert any systemic effect. The easy absorption, the long residence time, and the concentrations reached for those colonic metabolites in the plasma are the main features that support the possible bioactivity related to ingestion of ellagitannins.

In slaughtered Iberian pigs fed with acorns, urolithins start to appear in the jejunum [9], and 31 microbial ET-derived metabolites were identified in the plasma, urine, and bile, corresponding mostly to glycosylated and sulfated methyl glucuronides, glucuronidated conjugates of urolithins, and EA methyl ether glucuronides. In the case of humans, urolithins are probably synthesized in the large intestine, as healthy volunteers with ileostomies do not produce urolithins [60, 61]. In healthy humans, the maximum concentration of ETs metabolites is reached between 48 and 72 h, after ingestion of dietary ETs, but the persistence of urolithin derivatives in plasma and urine lasts until the fifth day [43], due to the enterohepatic recirculation [9, 54]. However, urolithin concentration in both plasma and urine after ingestion of dietary ETs varies considerably between individuals and depends on the assay conditions (i.e., amount of ET-rich food ingested, concentration and type of ETs, and composition of food matrix) [62]. In some healthy humans, urolithin A was found in plasma at concentrations as high as 18,6 μmol/L after the third and fourth day of pomegranate juice consumption [43], others reported an average of 1 μmol/L whereas other healthy human subjects were not able to release urolithins. In another study performed with 10 volunteers, urine was collected between 7 and 48 h after ET ingestion. 50% of the subjects excreted relatively high concentrations of urolithins, whereas others excreted very low amounts. In fact, one subject did not produce any urolithins during the study period [60]. These observations led to the concept of “high-

urolithin producer" and "low-urolithin producer". More than genetic factors, the high variability of physiological responses to dietary ETs was attributed to differences in gut microbiota ecology [17, 63, 64]. I*n vitro* anaerobic incubations of EA with fecal suspensions showed that, depending on the case, 80% of EA could be converted into urolithins, while others either did not produce urolithins or only traces after 72-h incubations [61]. In addition, variations between fecal suspensions were not only quantitative but also qualitative, as the profiles of conversion of EA to urolithins D, C, A, and B and other derivatives were markedly different [56, 61].

2.1.4 POTENTIAL IMPACT ON HEALTH

2.1.4.1 Reported Effect of Ellagitannins and EA.

Ellagitannins have been largely neglected in relation to their health benefits due to the high protein precipitation capacity associated to proanthocyanidins and gallotannins, in *in vitro* studies, but ETs have only a moderate one (e.g., Haslam et al., 1992; Kilkowski & Gross, 1999[65]. Regarding antioxidant activity ETs show the highest activity comparing to galloylglucoses, gallotannins and proanthocyanidins (condensed tannins) [21].

Despite their high antioxidant capacity [29, 66-69] ETs health benefits have been associated to other important biological properties, such as anticancer [70-72], anti-atherogenic, anti-thrombotic, anti-inflammatory, anti-angiogenic effects, [73], antihepatoxic, antibacterial and anti-HIV replication activities as mentioned in previous reviews [7].

Furthermore the hydrolytic derivatives of EA has also been extensively studied and associated to health benefits such as antioxidative, anti-inflammatory, anti-hyperlipidaemic and anticarcinogenic activities in a wide range of assays, both *in vitro* and *in vivo* [37, 53, 74-88], among other biological activities. However, given the metabolic studies that have shown the poor bioavailability of ETs and EA, it has been proposed that these compounds are able to exert only local health benefits in the colon, whereas the urolithins are the compounds that exert the most import health benefits such as prevention of cardiovascular diseases, anti inflammatory effects, chemoprevention of colorectal cancer, prostate and breast cancer under different mechanism of action. **Tables 2.4, 2.5, 2.6**, show the most

important results from intervention trials at clinical, animal and cell level, in which the health benefits attributed to ETs, EA and urolithins were evaluated.

2.1.4.2 Reported Effect of Urolithins.

Cellular models suggest that urolithins are active at physiological concentrations (4-18 μM) against chronic degenerative diseases; and health benefits have been proven on animal and cellular model studies. **Table 2.5,** and **2.6**

2.1.4.2.1 Chemoprevention of Prostate and Breast Cancers.

Cell models have shown urolithins as having mainly cancer chemoprevention effects, more specifically on prostate, breast, and colon cancers (**Table 2.6**). A major property of urolithins comprises their estrogenic (capacity to induce proliferation of MCF-7) and antiestrogenic (capacity to prevent or diminish the proliferation of MCF-7 cells in the presence of 1 pM estradiol) activities in binding estrogenic receptors, ER_β and ER_α. This may be due to the type and number of biophores in their molecular structures [89], giving them a structural analogy to estrogens [45]. Urolithin A, the main urolithin found in the circulatory system, was shown to have significant affinity for estrogen receptors, especially for ER_α. Urolithin B, the second metabolite of importance, showed lower but relatively high affinity for the estrogen receptors ER_β and ER_α [45, 50] than endogenous estrogen. As a consequence, urolithins strongly inhibit the growth of both androgen-dependent and androgen-independent human prostate-cancer cell lines (CaP). Urolithins appeared to inhibit the activation of the nuclear factor kappa B, which trigger the inhibition of sub-cellular pathways of inflammation, in CaP [23].

Table 2.4 Recent prospective clinical studies of consumption of food rich in ellagitannins

Ets Source	ETs intake	Status (Subjects number)	Duration intervention	Biomarker affected	Mains biomarker not affected	Ref
Pomegranate juice	Daily juice supplementation	Healthy(10)	1 and 3 years	Decrease in arotid intima-media thickness (IMT)		[90]
Pomegranate juice	50 ml daily	Healthy (10) non-insulin dependent diabetes mellitus (10)	3 month	Reduction of cellular peroxides (by 71%), and increased glutathion levels (by 141%). Decreased the extent of Ox-LDL cellular uptake (by 39%)		[91]
POMx capsules	1 capsule/days (410-850 mg GAE)	Overweight (64/32)	4 weeks	Increase in plasma antioxidant status	Glucose, BUN, creatinine, lipids, insulin, c-peptide, paraoxonase-1, or electrolytes or liver enzymes (AST or ALT)	[69]
Pomegranate juice	230 ml daily	patients with prostate cancer (24)	54 months	Significant reduction of prostate specific antigen		[92]
Commercial pomegranate extract in capsule	2*400mg in a single administration	Healthy (11)	24 h	+ 32% ORAC plasma antioxidant activity at 30 min	Generation of reactive oxygen species (ROS), inflammation marker interleukin-6 (IL-6)	[46]
Pomegranate juice	400 ml juice	Healthy	5 weeks	No benefit	Serobiochemical and haematological, urinary 8-iso-PGF(2 alpha), respiratory function variables and clinical symptoms of chronic obstructive pulmonary disease	[93]
Pomegranate juice	240 ml/day	patients with CHD and myocardial ischemia	3 month	Stress-induced ischemia decreased	No changes in cardiac medications, blood sugar, hemoglobin A1c, weight, or blood pressure	[94]
Pomegranate juice	50 ml/day	hypertensive patients	2 weeks	36% decrement in serum angiotensin converting enzyme (ACE) activity and a 5% reduction in systolic blood pressure		[95]
Mixed nuts	30g/day	subjects with metabolic syndrome	12 weeks	Increased excretion of serotonin metabolites		[96]
Strawberry	250 g daily	healthy	3 weeks	Lipid peroxidation lag time increased by 20%	DNA strand breaks in lymphocytes, and activity of phase II enzymes.	[97]

Table 2.5 Summary of intervention studies with animals to evidence health benefits of dietary ETs

Product ingested	Objective	Dose used	Results	Ref
Pomegranate extract	DU145, LNCaP, 22Rv1 LNCAP-AR cells Human prostate cancer cells (LNCaP) injected subcutaneously into mice	0.3 mg equ. punicalagin/mice	-Urolithins inhibited the growth of both androgen-dependent and androgen-independent prostate cancer cell lines, with IC50 values lower than EA. -EA and UA decreased prostate cancer xenograft size, tumor vessel density in 4 weeks	[98]
Raspberry juice	Heart health	Equ. 275 ml by a 70 kg human	Inhibited cardiac and aortic production of superoxide anion and increased hepatic glutathione peroxidase. lower plasma triglyceride level.	[99]
Blackberry extract	Antioxidant	2.68mg EA eq kg^{-1} body weight Wistar rat	Reduced thiobarbituric acid reactive substance levels and increased glutathione levels in the liver, kidney and brain	[100]
Berry juice and tea berry	atherosclerosis in hamsters	Equivalent to 275 ml by a 70 kg human	Inhibited aortic lipid deposition by 79–96% and triggered reduced activity of hepatic anti- oxidant enzymes after 12 weeks	[101]
Black raspberries extract	Anti-cancer	45 g per day	Inhibit esophageal tumorigenesis	[102]
freeze-dried berries	Esophagus and colon cancer	0.25-0.5 mg/kg body weight	Inhibition of rodent esophagus cancer by 30-60% and of the colon by up- to 80% Reduction of levels of carcinogen-induced DNA damage.	[103]
Purified Geranniin from *Geranuuim thunbergii*	Plasma antioxidant level	20 mg/rat	Increase plasma ORAC antioxidant value after 6 hours of ingestion	[104]
Lyophylized black raspberries, LBRs	Inhibition of *N*- NMBA-induced esophageal tumorigenesis in the rat during initiation and post initiation of carcinogenesis	0.25 mg/kg, weekly for 15 weeks and throughout a 30-week bioassay,	Reduced tumor multiplicity (39 and 49%, respectively). Inhibit adduct formation (64%) after NMBA administration at 0.50 mg/kg	[105]
		0.25 mg/kg, three times per week for 5 weeks	At 25 weeks, both 5 and 10% LBRs significantly reduced tumor incidence (54 and 46%, respectively), tumor multiplicity (62 and 43%, respectively), proliferation rates, and preneoplastic lesion development	
EA	mitochondrial damage in beta adrenergic agonist induced myocardial infarction	EA (7.5-15 mg/kg)/ 10 days	Protective effect of EA against mitochondrial damage in myocardial infarction	[85]
EA	Apoptosis	60 mg/kg body weight p.o. every day	Prevention of P13K-AKT activation in 15 weeks	[106]
EA	Anti-inflammatory properties	4 mg/kg rat	Significantly reduce paw edema	[77]
EA, robinetin, myricetin	Inhibitory effect on tumorigenecity of B[a]P 7,8-diol-9,10-epoxide-2 on mouse skin in the newborn mouse.	300 nmol	59- 66% Reduction in the number of skin tumors; after 15 and 20 weeks of promotion with 12-0-tetradecanoylphorbol-13-acetate	[76]
		45, 90, 180 nmol EA 200, 400, 800 nmol robinetin, myricetin	Avoid the formation of tumors in animals killed at 9-11 months later 44 — 75% inhibition in the- number of diol-epoxide-induced pulmonary tumors per mouse	
Pomegranate extract and urolithin A	Colon inflammation	250 mg PE/Kg day 50 mg UA/Kg day	Both PE and UROA decreased inflammation markers (iNOS, cycloxygenase-2, PTGES and PGE2 in colonic mucosa) and modulated favorably the gut microbiota, (increase in lactobacilli and bifidobacteria).	[107]
Pomegranate extract Urolithin A	Inhibition of tumor xenograft (LAPC-4) grow in immune compromised SCID mice	Orally / intraperitoneal PG: 0.8 mg/mouse/dose 5days / week UA: 0.3 mg/mouse/dose	Inhibition of 50% of tumor volume 2 weeks after	[108]
Urolithin A	Anti-inflammatory effect carrageenan-induced paw edema in mice	5 mg/mice	Reduction of paw edema after one hour of oral administration, increase of ORAC plasma level	[109]

Table 2.6 Summary of some recent results obtained on cell-models by ETs, EA and urolithins

Product tested	Cell-lines	Dose	Results	Ref
Punicalagin, EA	Colon cancer cell lines (HT-29 and HCT116)	100 µg/ml	Potent anti-proliferative activity	[68]
Pomegranate juice (PJ) total pomegranate tannins TPN Punicalagin (P)	Colon cancer cell line (HT-29)	50 mg/L punicalagin	Suppressed COX-2 expression by 97% (PJ); 55 % (TPN). 48% (P) respectively. TPT and P at 100 mg/L suppressed TNkF binding 10-fold and 3,6 fold respectively	[71]
Pomegranate juice extract	Prostate cancer		Regulation of androgen-independent prostate cancer cells	[110]
Raspberry extracts	Human cervical cancer (HeLa)	EC50= 17.5 µg/ml GAE	Inhibition of the proliferation of cancer cells	[111]
Berry extracts	Human oral (KB, CAL-27), breast (MCF-7), colon (HT-29,HCT116), and prostate (LNCaP), Caco-2	25 to 200 µg/ml	Dose dependant inhibition in all cell lines	[39]
Crude strawberry extract EA	Human oral (CAL-27, KB), colon (HT29, HCT-116), prostate (LNCaP, DU145)	100 µg/ml	Cell growth inhibition	[112]
ETs , EA	Caco-2 cells colon cancer	1-30 µM 1-100 µM	Apoptosis of cancer cells, Mitochondrial pathway, release of cytochrome C	[53]
EA	Colon, breast, and prostatic cancer	1-100 µM	Induce dose dependant apoptosis	[83]
EA	Colon cancer cells (SW480)	10M	Induce dose dependant apoptosis	[113]
EA	Human neuroblastoma (SH-SY5Y)	1-100 µM ; 96 h	growth inhibition, cell detachment and apoptosis	[114]
Punicalagin EA UA UB UC	PMA- cells (normal cells)	Punicalagin (IC_{50} =1.4 µM) EA(IC_{50} =1.1 µM) UA (IC_{50} =13.6 µM) UB($IC_{50=}$ 0.33 µM) UC (IC_{50} =0.16 µM)	Cellular antioxidant capacity	[14]
EA ETs UA	Colon cancer (293T)	ETs (IC_{50} =30.0 µg/ml) EA ($IC_{50=}$ 63 µM) UA (IC_{50} =39 µM)	Inhibited Wnt signaling Reduce colon carcinogenesis	[54]
UA UB UC	Prostate cancer	After 24 h UA (IC_{50} =13 µM) UB($IC_{50=}$ 17 µM) UC (IC_{50} =27 µM)	Inhibition of CYP1B1protein expression	[115]
EA,UA,UB	Breast Cancer	Significant inhibition when UB=5 µM	Chemoprevention of breast cancer. Inhibition aromatase activity	[72]
EA	Pre-B acute lymphoblastic leukemia (ALL) cell lines	100 µg/ml	Induce apoptosis in high-risk leukemia cells	[116]
EA, UA UB	Colon cancer Caco-2	40 µM	Chemopreventive effects, modulate phase I and phase II detoxifying enzymes	[75, 117]
UA, UB	Breast cancer (MCF-7)	IC50 ERα and ERβ binding assays were: 0.4 and 0.75 µM for UA; 20 and 11 µM for UB	Estrogenic antiestrogenic activity	[45]
UA; UB, MUA, MUB; UBS; acetylated-UB	Breast cancer	UB highest at 2;35 :4,7 µmol/L	UB Inhibit significantly aromatase activity and testosterone-induced MCF-7aro cell proliferation	[72]
EA	Cervical cancer	10 µmol/L	Inhibit cell growth and induce apoptosis	[79]

Urolithins were also found to inhibit activity of the enzyme CYP1B1. This inhibition may affect the three stages of prostate cancer development. Urolithins therefore, appear to display a dual mode of action—inhibiting the activity and/or expression of the enzyme. Urolithin A and B inhibit CYP1 E ROD activity by inhibiting both the protein expression and the activity of CYP1B1; and thus decreasing the incidence of prostate cancer [118]. In addition, if used as an adjuvant during chemotherapy, these microbial metabolites appear to decrease the resistance to the drug mediated by CYP1B1.

Urolithin A glucuronide and, in a much lesser amount, dimethyl ellagic acid were reported as accumulating in human prostate tissues after subjects had consumed pomegranate juice [50]. Subjects who had received the primary treatment of surgery or radiation also demonstrated significant decrease in prostate-specific antigens (PSA) after consuming pomegranate juice [23, 92]

All these findings, along with animal studies [98, 108] (**Table 2.5**), justify the high interest in ET-rich diets as preventing prostate cancer. Less information has been published so far on the urolithins' role in reducing risks of breast cancer, although they have been shown *in vitro* to significantly enhance the apoptosis of breast-cancer cell lines [45, 71, 72] (**Table 2.6**).

2.1.4.2.2 Chemoprevention of Colorectal Cancer.

Cell models have shown that urolithins induce dose-dependent apoptosis of colon-cancer cells [54, 75, 117]. Urolithins A and B appeared to modulate phases I and II detoxifying enzymes in Caco-2 cells [117]. Both phase enzymes enhance the detoxification of carcinogenic compounds. Another study proposes an additional pathway by modulating the expression levels of multiple genes in the epithelial cells lining the colon. This pathway might be used by EA and urolithins A and B, thereby exerting an effect towards reducing risks of colon cancer [75]. This study suggested the urolithins' antiproliferative activity may be due to the modulation of gene expression, including the MAPK/ERK pathway signaling. Urolithin A can induce cell cycle arrest, and modulate key cellular processes associated with colon cancer development, such as MAPK signaling *in vitro*. Additionally, urolithin A was shown to inhibit Wnt signaling with IC_{50} at even lower concentrations than for EA [54].

2.1.4.2.3 Anti-Inflammatory Effect

The inhibition effect of urolithins on cancer may also be due to the anti-inflammatory activity, as urolithin A was also found to decrease inflammatory biomarkers in a colitis rat model, including nitric oxide synthase, cyclooxygenase-2 (COX-2), prostaglandin E synthase, and prostaglandin E2 [107]. Oral ingestion of EA and urolithin A has also been shown to quickly and significantly decrease inflammation in the rat's-paw edema model [77, 109]. This anti-inflammatory effect was also observed in a clinical study on both healthy and non-insulin-dependent diabetic patients consuming pomegranate juice, which greatly reduced cellular peroxides and increased glutathione levels [91] after 4 weeks of daily intake. However, another study using a much shorter period (24 h) found that inflammation biomarkers such as interleukin-6 remained unchanged [46].

2.1.4.2.4 Prevention of cardiovascular disease (CVD)

Consumption of ET-rich food has also been associated with cardiovascular health. Clinical studies have demonstrated inhibited lipid peroxidation [91, 97]; antiatherogenic activity [119]; reduced stress-induced ischemia [94]; reduced systolic blood pressure [95]; reduced platelet aggregation [120]; and decreased carotid intima-media thickness and angiotensin-converting enzyme, which are linked to artery distensibility [90, 95].

2.1.4.2.5 Antioxidant activity of urolithins

Contrary to what was previously shown by strictly chemical antioxidant assays [43, 121], cell-based assays showed that the urolithins' antioxidant activity correlated with the number of hydroxyl groups and molecule lipophilicity. The most potent antioxidant urolithins were C and D, with IC_{50} values, respectively, at 0.16 and 0.33 μM, followed by urolithin A with an IC_{50} value of 13.6 μM. Urolithins C and D presented lower activity than their parent molecules—respectively, EA and punicalagin—which had IC_{50} at 1.1 and 1.4 μM, respectively [14]. The monohydroxylated urolithin B and the derived methylated compounds did not show antioxidant activity.

Although urolithin A presented the weakest antioxidant activity, its IC_{50} remained at physiological concentration, at least for high-urolithin producers, and thus may potentially contribute to *in vitro* antioxidant capacity. However, antioxidant capacity is apparently not a major property of the most bioavailable ET metabolites (urolithins A and B), at least, not in chemical or *in vitro* cell-based tests. Clinical studies showed a

slight increase in the antioxidant capacity of plasma after long-term consumption of ET-rich food [46, 69, 97], whereas another study failed to show this [93]. Again, the high variability of physiological responses in human subjects during clinical studies is not conclusive. Their nature as a "low-urolithin producer" or "high-urolithin producer" should be taken into account to better demonstrate the in-vivo antioxidant potential of urolithins.

2.1.5 TOXICITY OF DIETARY ELLAGITANNINS

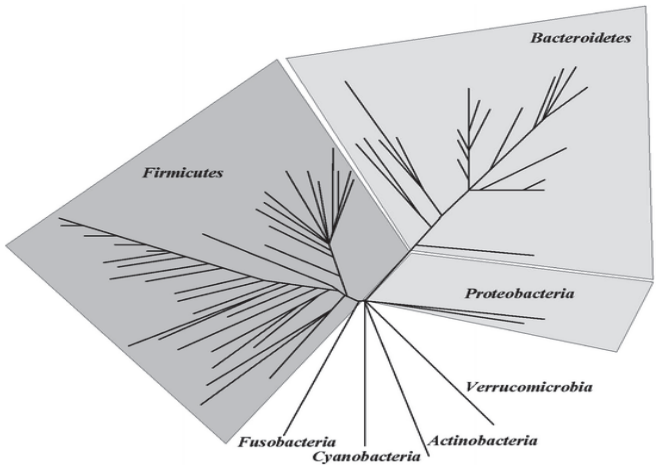
Various pharmacokinetics and toxicity studies have proven that ET-rich foods are not toxic even when consumed in large amounts [122, 123]. For example, a daily diet of 30% oak-flavored milk powder, containing large quantities of ellagitannins, was not toxic to rats for almost 100 days. Urolithin A and its glucuronide were detected in feces and urine, but no accumulations were found in the liver or kidneys, as corroborated by histopathological analyses. Nor were accumulations found in the uterus. Neither did the diet change common biomarkers in the blood [124]. Another study, using high doses of pomegranate in the rats' diet, had similar results, except for urea and triglycerides, which remained low throughout the experiment, because the diet was of lower nutritional value than standard rat food [49]. Clinical trials on overweight human subjects indicated that ETs as a dietary supplement are safe for human, with up to 870 mg of gallic acid equivalents per day (i.e., equivalent to about 8 liters of pomegranate juice per day) over 1 month [69].

2.2 GUT MICROBIOTA AND BIOCONVERSION OF DIETARY COMPOUNDS

Human colon harbors complex microbial ecosystems distributed along the entire GI tract and integrated by more than 800 different bacterial species [125, 126], that include species of resident microflora and transient microbes from food- and water-borne sources [127]. Density and diversity of gut microbiota increase from the stomach to the colon [126, 128]; and this is strongly affected by intestinal transit time [129]. Microbiota composition is claimed to be individual-specific and influenced by genotype, diet and lifestyle among others, [1, 62, 130]. Colonic microbiota is dominated by strict anaerobes, which are present in 1000-fold higher than facultative anaerobes [16, 131].

The restricted access to the different compartments of the GI tract and the difficulty to grow gut bacteria by classical means has limited their study, while the knowledge about microbiota composition and functionality is quite scarce in scientific literature. For many years, only faecal culture methods were used to study gut microbiota composition, consequently the studies were biased as they quantified only culturable bacterial species; and besides faecal samples are not representative of bacterial communities inhabiting the caecum and ascending colon [131].

Fortunately, recent advances in independent culture methods based on the sequence variability of the 16S rDNA genes have led to identify the most common bacterial phyla inhabiting the gastrointestinal tract [132]. From culture studies, it has been pointed out that the *Firmicutes* phylum is the most common bacterial species present in the gut, with 208 members identified, followed by *Gamma proteobacteria* with 67 members [133]. However from the subunit ribosomal RNA (SSU rRNA) study, 301 members of *Firmicutes* phylum were identified, while from the *clostridium* phylum 174 members were identified [126, 134]. **Figure 2.2** shows a proposed phylogenetic tree of the human gastrointestinal microbiota composition.



A recent study based on molecular methods and conducted on a large population in different European countries suggested that all humanity could be clustered in three main enterotypes according to the predominant bacterial flora in their gut, *Ruminococcus, Bacteroides* and *Prevotella* [135]. In this study, it was shown that on

average 50% of Europeans belong to the group where *Ruminococcus* prevail in microbiota, 30% to *Bacteroides* and 20 % to *Prevotella*. Additionally, these specific groups were apparently associated with specific diet, *Bacteroides* species being more common in people consuming more meat proteins, and *Prevotella* associated to vegetarian diets.

Further advances in the characterization of taxonomic composition, metabolic activity, and immunomodulatory capacity of the human gut microbiota, has led to assess its role in human health and disease [136]. The gut microbiome (microbial genome) represents therefore a gene number that is 150 times greater than the human genome [42]; and thus encode a spectrum of metabolic capabilities beyond that of the host genome, whereby the microbiota can be considered as a relevant component of human physiology [136].

The human-microbial ecosystem comprises a considerable metabolic versatility, using biological pathways that humans are not able to do [15]. Growing evidence suggests that gut microbiota is one of the most important factors contributing to the host response towards nutrients [42]. The gut microbiota interacts symbiotically with the host with an unlimited metabolic potential [129, 133, 137]. The host-microbiota relation is a homeostatic symbiosis, in which the host provides the microbiota with nutrients and a stable environment, while microbiota may modify epithelial expression of genes involved in many different functions concerning immune or neuroendocrine system, metabolism, or general cellular functions among others [1, 62]. The gut microbiota exhibit strong metabolic activity at different extension, as it is involved in the catabolism of a vast range of dietary and endogenously secreted compounds, participating in the control of energy regulation, nutrients metabolism and transformation of xenobiotics. [125].

The gut microbiota is able to convert dietary compounds that are not bioavailable as such into lower-weight metabolites that can be absorbed. Microbial enzymes can hydrolyze glycosides, glucuronides, sulfates, esters, and lactones and operate ring-cleavage, reduction, decarboxylation, demethylation, and dehydroxylation reactions[137]. Although information is still scarce in this thriving research field, **Table 2.7** summarized the main studies on known dietary compounds metabolized by gut microflora into more bioactive compounds.

Butyrate was the first colonic metabolite observed that comes from the ingestion of dietary fibers. A wide variety of butyrate-producing bacteria belonging to the *Firmicutes* phylum have been identified. Butyrate is the main source of energy of colonocytes, and its bioactivity is specially related to the health state of the colon [131, 138].

Conjugated linoleic acids (CLAs) are other colonic metabolites that have been studied. CLAs represent a group of isomers of linoleic acid released by a combination of *lactobacilli* and *bifidobacteria* using linoleic acid as substrate [131]. The bioactivity of these metabolites is high, and includes anti-inflammatory and cancer-preventive properties, decreasing cancer cell viability and inducing apoptosis [139].

Table 2.7 Main studies on bioconversion of dietary compounds into bioactive colonic metabolites [131]

Dietary component	**Food Source**	**Colonic microbiota metabolite**	**References**
Dietary Fibers	Grains or derivatives	Butyrate	[138-146]
Linoleic acid	Vegetable oils	Conjugated Linoleic Acid, CLA	[147-149]
Daidzein	Soy	Equol	[150-160]
Secoisolariciresinol	Flaxseed, sesame	Enterolactone, enterodiol	[161-167]
Isoxanthoumol	Hops/hop-derived products	8-PN	[168-171]
Ellagic acid	berries, walnuts, pomegranate	Urolithins A and B	[14, 45, 50, 54, 75, 107, 115, 117, 172-176]

Equol is another colonic metabolite produced during the metabolism of daidzein, the main isoflavones in soy beans. However, it was shown that only 30% of humans are able to transform dietary isoflavone daidzeine into equol [152]. Equol is a bioactive compound with anti-androgenic activity [177] and strong estrogenic activity which means that equol producers have a lower risk of developing prostate and breast cancer [178].

These equol studies revealed for the first time the importance of gut microbiota in human health, as japanese women were able to take more advantages of the hormone substitutive therapy treatment by ingesting soy derived food while it was mostly ineffective in their European counterparts. In fact, japanese women were more able to convert soy isoflavone into equol, the bioactive colonic metabolite. Intensive research led to the identification of some gut microorganisms able to perform such

bioconversion. More recently, it was shown that equol production was positively correlated with the abundance of sulfate-reducing bacteria, and negatively correlated with *Clostridium coccoides–Eubacterium rectale* counts [168]. Other studies have been carried out but were unsuccessful in identifying the microorganisms responsible for daidzein bioconversion [152, 178-180]. Finally, a NATTS strain, a Gram-positive, non-sporulated rod bacteria, belonging to genus *Slackia* (Actinobacteria) was found to convert daidzein into equol [181]. Additionally, anaerobic incubation mixture of two bacterial strains *Eggherthella* sp Julong 732 and *Lactobacillus* sp. Niu-O16 were also able to release equol *in vitro [180]*. Other investigation reported that strains FJC-A10 and FJC-A161 could metabolize dihydrodaidzein to equol along with Julong 732 [179].

Plant lignans such as secoisolariciresinol ingested with beer, for example, are also a substrate for gut microbiota, which is able to metabolize them into enterodiol and enterolactone. Conversely to daidzein the bio-conversion of secoisolariciresinol to enterodiol and enterolactone is common in most studied human subjects. Eleven bacterial species involved in the metabolism of secoisolariciresinol diglucoside have been isolated so far from human feces. The bioconversion of the dietary lignan secoisolariciresinol diglucoside (SDG) via secoisolariciresinol (SECO) to the enterolignans enterodiol (ED) and enterolactone (EL) is carried out by phylogenetically diverse bacteria. Strains of *Bacteroides distasonis, Bacteroides fragilis, Bacteroides ovatus*, *Clostridium cocleatum*, an isolated strain Clostridium sp. SDG-Mt85-3Db, are as well as strains of *Butyribacterium methylotrophicum, Eubacterium callanderi, Eubacterium limosum, Peptostreptococcus productus*, *Clostridium scindens* and *Eggerthella lenta* strains are involved. The dehydrogenation of ED to EL is carried out by strain ED-Mt61/PYG-s6. [162]. Lignans have showed estrogenic, hypocholesterolemic, antiatherogenic and antioxidant activities, which can be considered as protective against breast and prostate cancer, coronary and heart disease [161, 163, 164].

Prenylfavonoids, (xanthohumol, isoxanthohumol) and 8-prenylnaringenin (8-PN) are other groups of compounds present in hops and derived products that were shown to be bioconverted by gut bacteria. 8-PN is a bacterial metabolite resulted from isoxanthohumol biodegradation [171]. Like equol, 8-PN apparently occurs in only one third of humans. In this case *Eubacterium limosum* appeared to be involved in the

conversion [182]. In contrast, 8-PN is one of the most potent phytoestrogens but is less effective than xanthohumol in inhibiting growth of colon cancer cell lines [170].

Nonetheless, the current knowledge on the interaction between dietary compounds and gut microbiome, enzymes and metabolic pathways involved is still scarce and limited to food products with huge commercial interests such as soybean and beer. Studies on the bioconversion of ellagitannins and ellagic acid have only emerged very recently and no information is available yet about the species of colonic microorganisms that could be involved in this bioconversion process.

3 MATERIALS AND METHODS

3.1 REAGENTS AND MATERIALS

3.1.1 CHEMICALS

Urolithin A (3,8-dihydroxy-6H-dibenzo[b,d]pyran-6-one; >95% purity) and Urolithin B (3-hydroxy-6H-dibenzo[b,d]pyran-6-one; >98% purity) were purchased from Kylolab (Murcia, Spain). Ellagic acid was acquired from Sigma Aldrich (St. Louis, MO, USA). Methanol, formic acid and acetonitrile were obtained from Merck (Darmstadt, Germany). Ascorbic acid and β-glucuronidase from bovine liver (1 MU units /g) were obtained from Sigma Aldrich (Steinheim, Germany). Milli-Q water (Milli-Q system, Millipore Corp., USA) was used for these assays.

3.1.2 GROWTH MEDIUM

Brain heart infusion (BHI) medium was obtained from Fluka, Sigma Aldrich chemie GmbH, and resazurin sodium salt was obtained from Sigma-Aldrich (Steinheim, Germany). L-cysteine HCL was provided by Sigma Aldrich (Steinheim, Germany) Milli-Q system (Millipore Corp., Bedford, MA) ultrapure water was used throughout this experiment.

3.1.3 BLACKBERRY JUICE, BBJ

Ripe blackberries (*Rubus adenotrichos*) cultivated to 1800 m.a.s.l. were purchased from a tropical highland blackberry growers cooperative (APROCAM, Cartago, Costa Rica) certified in organic farming. Blackberries were pressed in a hydraulic press (300 kPa) to extract the juice, followed by one hour of enzymatic treatment with a mixture of cellulase and pectinase (200 ppm at 30°C with Ultrazym AFP-L®, Novozyme, Denmark) and microfiltration on a ceramic membrane (Membralox IP19-40, PALL corporation) with 0,2 μm pore diameter. The recovered permeate was packed in triple-laminated 250-mL aluminum bags and frozen at -20 °C. Then, BBJ was directly filtered through a 0,45 μm filter and analyzed by LC-MS.

3.2 ANALYTICAL METHODS

Liquid chromatography and mass spectrometry were the methods chosen to analyze urine and broth cultures looking for ETs metabolites that shed lights on the metabolic pathway followed by blackberry ellagitannins once BBJ is ingested.

3.2.1 SAMPLE PREPARATION

3.2.1.1 Urine preparation

For LC-MS urine analyses, the sample preparation can be limited to centrifugation in order to remove particulates, and subsequently dilution with water (1:1 to 1:3 vol/vol) in global metabolites analysis to minimize potential analyte losses [183]. However in a target metabolic profile, metabolites can be isolated by simple methods such as solid phase extraction (SPE), solid phase extraction chromatography (SPEC) or preparative HPLC. SPE is an efficient selective isolation multistage separation technique, and even though is a time-consuming multi-step process, it was the method chosen to prepare the urine for further analysis. Therefore, a SupelcleanTM LC-18 extraction cartridges (reverse phase Supelco Analytical USA) were activated with 3 mL of MeOH and 10 ml of water, and subsequently a 10-mL urine sample was carefully added. After the total sample had passed through the cartridge, Milli Q water (3x3 mL) was added to wash the cartridge and remove all the water soluble components. Then MeOH was added (3x1 mL) to remove the target compounds. Each methanolic fraction was recovered and filtered through a 0.45 μm membrane filter (Waters Millipore) and then analyzed independently for ET-metabolites.

Enzymatic treatment. Some urine samples were treated with 40 units/mL of β-glucuronidase as described previously [9] to evidence conjugation of UA and UB to glucuronides moieties. One ml of urine was added with 144 μL of acetate buffer (0,1 M, pH 5) and the enzyme was incubated overnight. Then, the samples were extracted with diethyl ether three times. The organic phases were pooled and evaporated under reduced pressure until dry and re-dissolved in methanol, it was then filtered through a 0,45μm membrane filter to be further analyzed. All methanolic fractions were analyzed by UPLC-DAD/ESI+-Q-TOF/MS for 21 compounds previously reported as ETs metabolites.

3.2.2 ANALYSIS PERFORMED FOR THE IDENTIFICATION AND QUANTIFICATION OF ETs AND THEIR METABOLITES

Despite important recent advances in analytical science, the detection of all types of molecules using a single technical principle is not possible. Hyphenation of different techniques allows exploiting the advantage of different analytical principles. Hence, we combined chromatographic and spectral methods for the identification and quantification of ETs and their metabolites. Chromatography allows the separation of nearly pure fractions of chemical components in a mixture; and, mass spectroscopy produces selective information for their identification using commercial standards or library spectra.

3.2.2.1 Principle of chromatographic separation

Liquid chromatographic separation is defined as the separation of the components in a mixture, based upon the rates at which they elute from a stationary phase over a mobile phase gradient. Molecules dissolved in the mobile phase are separated depending on their distribution coefficient in the two phases, while the mobile phase passes through the stationary phase [184]. There are two different phases: the Normal phase in which the stationary bed is strongly polar and the mobile phase is largely non-polar; and Reverse phase where the stationary phase is non-polar and the mobile phase is polar. Their typical surface coatings are -Si-OH, $-NH_2$ for normal phase and C8, C18, Phenyl for Reverse phase, $-NH_4$ for anion exchange and –COO- for cation exchange. Elution can be carried out at isocratic (fix concentration) or gradient (eluent concentration and strength are changing). Most LC instruments are equipped with an optical detector: universal such as refractive index RI, evaporative light scattering detector (ELSD), or selective such as UV/VIS light, Fluorescence and Electrochemical, photo diode array detector (PAD) and finally, Mass spectrometer MS.

Ultra performance liquid Chromatography (UPLC)

Recent advances in instrumentation and column technology [185] have led to an important increase of separation efficiency, resolution, speed and sensitivity. These important improvements gave origin to Ultra performance liquid Chromatography which used shorter columns with superior mechanical strength, high pH stability, smaller

particle size (<1,7 μm) and high pressures (>15000 psi). The ultra-efficient UPLC columns overcome peak shape asymmetry and poor loading capacity for basic compounds, column bleed, and rapid mobile-phase re-equilibration. **Figure 3.1**, illustrates in a 3D map the differences between HPLC-MS and UPLC-MS analysis in terms of numbers and intensity of peak when analyzing mouse urine samples. **Figure 3.2**, depicts the differences concerning speed, sensitivity and resolution between HPLC and UPLC analysis of the same sample.

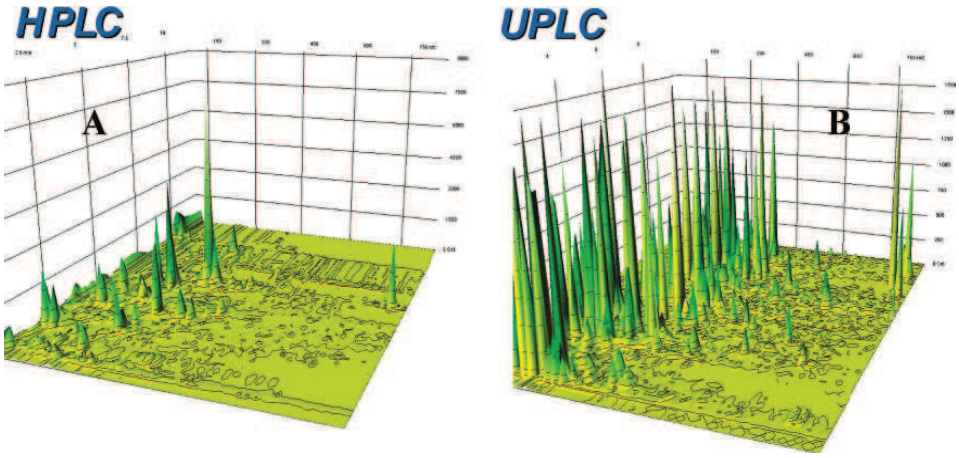


Figure 3.1 HPLC-MS and B) UPLC-MS analysis of mouse urine in a 3-dimensional map to observe the differences on the information generated [1]

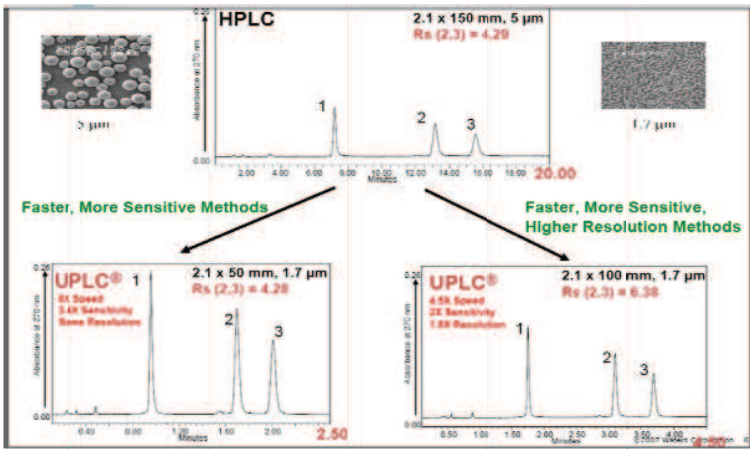


Figure 3.2 Comparing speed, sensitivity and resolution in HPLC and UPLC analysis [2, 3]

Thus, as UPLC/MS results in a well-suited tool to approach efficiently metabolite detection and eventually identification [186], it was the method selected to carry out the separation and identification of ETs metabolites in the present study; whereas for ETs content in BBJ, HPLC was the method followed as it is the most common method in the characterization of ETs in foods [7, 25, 29, 31, 47].

3.2.2.1.1 Chromatographic Separations of Ellagitannins in Blackberry Juice

Microfiltered blackberry juice was analyzed on a HPLC-DAD. The HPLC system consists of a Shimadzu SPD M20 diode array detector, a Shimadzu LC 20 AT pump, a Shimadzu DGU 20A5 degasser and a Shimadzu oven for the CTO 6A column. The BBJ analysis was performed on a reversed-phase C18 column (Phenomenex prodigy, 5 µm ODS C18, 150 mm x 2.00 mm, 100 A), at 30 °C. Solvents A and B were H_2O/HCO_2H (98/2; v/v) and CH_3CN/ H_2O/HCO_2H (80/18/2; v/v/v), respectively. The gradient started with 5% B in A to reach 25% B in A at 20 min, 100% B in A at 25 min, 10% B in A at 30 min, and 5%B in A at 35 min. The most representative ETs from blackberry juice were identified according to their retention time and their UV spectra.

3.2.2.1.2 Chromatographic separation of ETs metabolites

Although there are many factors that affect performance of the analysis, the gradient and the stability of the column play a leading role. In chromatographic separations the gradient is sample dependent, but in general UPLC gradients for urine samples it can vary from 5 to 30 min. Nevertheless, short runs will detect fewer ions than long ones. A good balance between speed of analysis and more exhaustive coverage of urine metabolome was set between 10–12 min. The gradient started with aqueous solutions (99–100% water), and change until reaching 95–100% acetonitrile or methanol. The column was washed to remove the salts and unwanted contaminants to prevent deterioration of the column. After the wash period, start conditions are resumed to ensure the column re-equilibration. Five column volumes of initial solvent were used before injecting the following sample. To ensure repeatable results concerning retention time RT, the column signal intensity was stabilized by injecting 5 to 10 times the matrix (urine or extracts). This reduces the variability along of the run. Consequently, running at least 5 pooled quality control QC samples at the beginning and at the end of the pool samples analysis allows the gathering of information about the stability and suitability of the system.

Afterwards, each run column was washed with a strong eluotropic solvent to avoid the accumulation of contaminants and guarantee continuing good performance. [183]

- Specific conditions for urine analysis

The components of interest in urine are predominantly of low molecular mass and are generally hydrophilic, thus reverse phase columns, typically C18-bonded silicas, provide a good general system for the metabolite profiling of urine. Chromatographic separations of ET-metabolites were carried out in a Waters Acquity Ultra Performance Liquid Chromatography system UPLC equipped with a Photo diode Array Detector PDA, a binary solvent manager, autosampler, and degasser controlled by software from Waters (MassLynx V4.1) (Waters Corp., Milford, MA, USA). The column eluent was directed to the mass spectrometer.

Metabolite separations from urine were carried out in a reversed-phase ACQUITY UPLC C-18 CSH (Ireland) 100* 2.1 mm with a 1.7μm particle size and a precolumn (2.1 mm, 0.2-μm particle size) that was maintained at 30 °C. Solvent A consisted on Water/formic acid (99.9:0.1, v:v) while Solvent B consisted on Acetonitrile/formic acid (99.9/0.1 v/v). The flow rate was 0.400 mL/min, and the injection volume was 6 μl. The gradient for urine samples started with 100% A to reach 10% B in A at 7 min, 95% B in A at 22 min and 100% A at 22.1 min. The gradient was stabilized at 100% A at 26 min. The ACQ-PDA was operated at a sampling rate of 20 points/s, and ranged from 190 to 600 nm. The column eluent was directed to the mass spectrometer.

- Specific conditions for analysis of extract from colonic fermentation broth

Metabolite chromatographic separations from a broth of batch culture incubation were performed on a reversed phase C-18 column ACQUITY UPLC BEH 1.7 μm * 2.1* 50 mm (Ireland) maintained at 30 °C., using water/formic acid (99:1, v:v) as solvent A; and MeOH as solvent B. The flow rate was 0.208 mL/min, and the injection volume was 1.0 μ. The gradient for elute ET-metabolites started with 20% B in A to reach 70% B in A at 5 min, 90% B in A at 6 min and 95% B in A at 7.2 min, then return to 20% B in A at 7.4 min and was stabilized in that condition until it reached 9 min. The ACQ-PDA was operated at a sampling rate of 20 points/s and ranged from 200 to 400 nm.

3.2.2.1.3 Quantification of Ellagitannins (ETs) and their Main Metabolites

Quantification of ellagitannins and their metabolites was done using chromatographic analysis with a photo diode array detector (PDA). ETs were quantified as free EA equivalent and expressed in µmol/L of EA equivalent. The UV spectrum was recorded at 280 nm and the ETs concentration was determined from the peak area by using the equation for linear regression obtained from the calibration curve of EA, **Figure 3.3,** obtained from authentic standards of ellagic acid (EA)

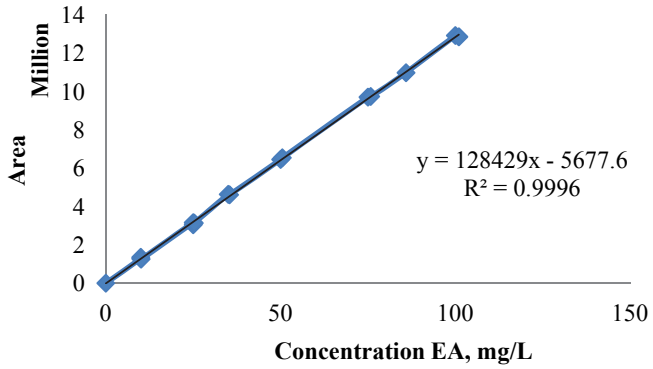
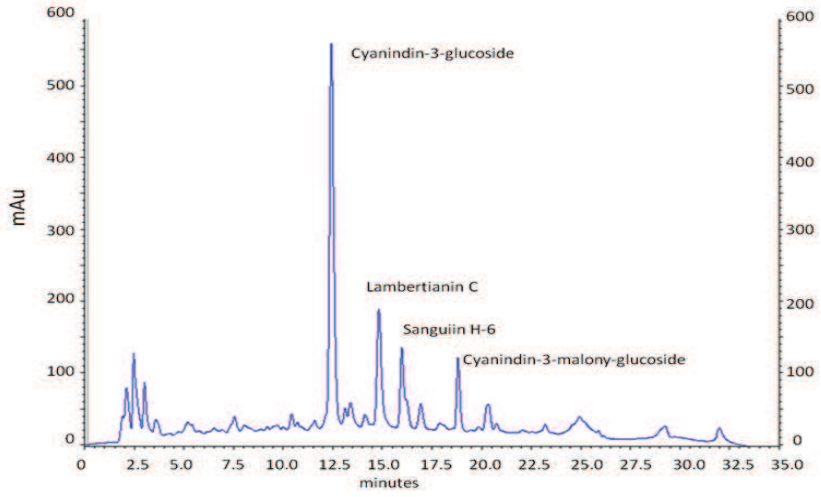


Figure 3.3 Calibration curve of EA, for quantification of ellagitannins by HPLC, following the method proposed by Mertz, 2007.

The limits of detection and quantification were 1.6 and 5.5 mg/L of EA respectively. **Figure 3.4**, illustrates chromatograms from the BBJ, where the main anthocyanins and ellagitannins of blackberry (*Rubus adenotrichos*) can be observed.



UA, UB, UC, UD, metabolites including glucuronide forms were quantified recording the area under the curve of UV spectra at 278 nm and concentration was always reported in UA or UB equivalent. A calibration curve with UA and UB standards, **Figure 3.5** was done before each analysis always with R^2>0.97. In the absence of standards for glucuronide UA and UB they were always expressed as µmol UA and UB equivalent. An example of the data provide by the UPLC analysis is depicted in the **Figure 3.6**.

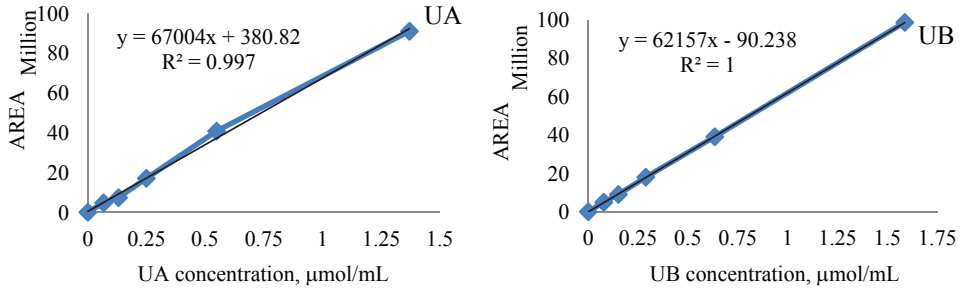


Figure 3.5 Calibration curve of EA, UA and UB for quantification of ellagitannins in BBJ, and ETs metabolites in urine and broth culture incubation.

3.2.2.2 Mass spectrometry analysis.

High resolution mass spectrometry aim at measuring the accurate mass of ions produced after ionization of compounds. Therefore, there are at least three main components of a mass spectrometer: an ionization source, a mass analyzer and a detector.

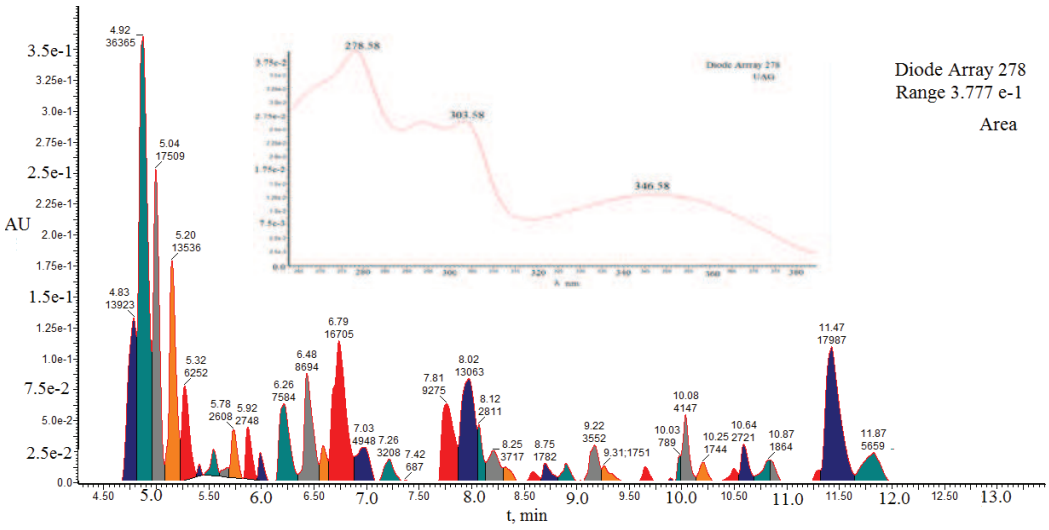


Figure 3.6 Basic information provided by UPLC: UV chromatogram-Area under the curve and the UV spectra for m/z+ 213.0552 ion is illustrated

3.2.2.2.1 Ionization Source

The aim of ionization is to convert molecular compounds into ions volatilized in a gas phase. Different ionization techniques are used, such as elestrospray ionization (ESI), Atmospheric-pressure chemical ionization (APCI), Electron ionization (EI) and matrix assisted laser desorption/ionization (MALDI), allows the conversion of most large, non-volatile and thermally labile compound into a gas-phase ion.

The electrospray ionization (ESI) is a soft technique often preferred in hyphenated LC-MS techniques since it produces larger numbers of ions [187]. The development of ESI has been an important advance as it allows desorption and ionization of a wide range of molecules directly from the mobile phase proceeding from LC [188].

ESI process is depicted in **Figure 3.7**, in which ions are generated at atmospheric pressure after passing through a capillary with and inner diameter >250μm and being sprayed in a ionization chamber where there are voltage differences between 500 and 4500 V. The inner diameter of the needle and the solvent used define the voltage required. At the end of the capillary, an aerosol of charged droplets is generated. Depending on the polarity of the voltage applied, the droplets of solvent and analyte can be negatively or positively charged [189]. The solvent evaporates and the droplets shrink until the charge density of the surface prevails over collusion forces, giving rise to repulsive forces between charges that lead to drop fission and expulsion of the formed ions.

ESI works best with solutions that have a high percentage of organic solvents, such as acetonitrile or methanol, even though the solution should remain partially aqueous with some ions such as hydronium, hydroxyl and sodium ions. Positive electrospray ions are produced by adding of a positive molecule, called adduct which nature depends of the salts present in the medium (generally H^+, $NH4^+$, Na^+). Negative electrospray ions are generally produced by the removal of a proton H, but other adducts with Na-2H and Cl, can also be produced.

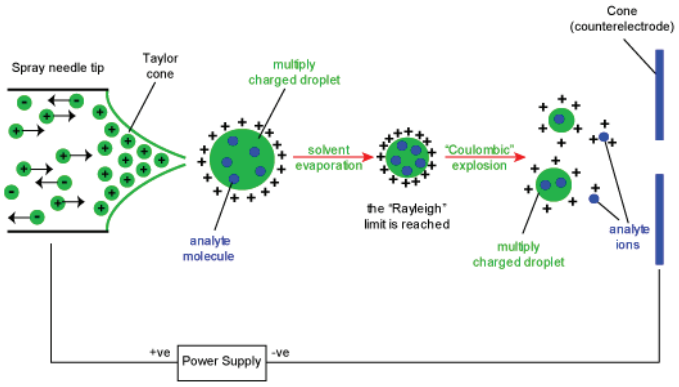


Figure 3.7 Electrospray ionization principles[190]

Some analytes are ionized more efficiently in one ionization mode rather than in another mode (**Table 3.1**), for different and often complex reasons. Usually metabolites containing only C, H and O can be detected in negative ion mode, but if they also contain N, ionization in positive-ion mode is recommended [183]. Therefore, during metabolomic studies, usually, ionization is carried out in both positive and negative mode, enabling the detection of two sets of analytes which may differ significantly. Concerning our targeted analysis, ESI in positive mode (ESI^+) was the method applied for the analysis of ETs metabolites in urine and fermentation broth. ESI^+ showed higher signals than ESI in negative (ESI^-) mode in preliminary studies. Additionally, formic acid was added to the mobile phase to obtain a stronger signal.

Table 3.1 Relative efficiency of electrospray ionization of common chemical functional groups (Fiehn Laboratory, UC Davis)

	Positive ionization	Negative Ionization
Decreasing efficiency	Quaternary ammonium Amines	Sulphates and phosphates
	Amino acids	Sulphonates, phosphonates
	Ketones, aldehydes	Carboxylates
	Esters	Peroxide
	Carboxylates alcohols	Amino acids
		Thiols, alcohols

The main concern with ESI is its tendency to ion suppression or enhancement phenomenon, which can lead to adverse effects during quantification. Matrix effects can cause ion suppression and a compound may be much more efficiently ionized than others being quantitatively over estimated whereas others are under estimated [184]. For example, salts and non-volatile solutes can change the efficiency of droplet formation and evaporation which changes the amount of charged ion in the gas phase that reaches

the detector [191] yielding a mass spectrum with peak intensities not proportional to the real concentrations found in the sample. Hopefully, targeted analyzed compounds showed a significant trace in UV spectra, which allowed a more reliable quantification by these means. A semi-quantitative analysis was performed, referring to any relative changes of the same ion peak intensity in the same urine matrix, supposing that ion suppression or enhancement was similar in the different sample. Actually, this is the main principle that prevails during untargeted metabolomic analysis where changes of the same ion peak intensity are observed in samples from very similar matrixes.

The other existing ionization techniques are more severe. For instance, EI which ionized molecules by a beam of high energy electrons creates ions by collisions which often cause fragmentation of parent molecules. In most cases, fragments produced in standard conditions (at 70 V) can be used to identify metabolites, as extensive libraries exist. Generally, EI is limited to the analysis of small volatile compounds. In APCI, the compound is heated by a ceramic tip up to 500°C, with cross flow of nitrogen, in order to reduce the droplet size. One of the most important advantages of APCI over ESI is that it is less susceptible to matrix interferences. In addition, APCI can ionize weak polar compounds, but one major disadvantage is the ionization of labile compounds, which can be decomposing because of the high temperatures reached during nebulization (400-500°C) [189]. The last technique, MALDI is also more appropriate for thermolabile, non-volatile, organic compounds with high molecular mass. In this case, the sample is bombarded with a pulsed laser light, then, the sample is co-cristallized with a solid matrix that absorbs the light emitted by the laser. Analytes are fixed in a chemical matrix that absorbs energy from an ultraviolet laser, resulting in an analyte desorption and ionization. [192]. MALDI is widely used in proteomics and for oligosaccharides, and oligonucleotides among others.

3.2.2.2.2 Mass Analyzers and Detector

In the market, a large number of mass analyzers are currently available, but almost all of them use at least one or a combination of the following principles: quadrupoles, time-of-flight (TOF), and Fourier transform Mass spectrometer.

- Quadrupole. A quadrupole mass analyzer consists of four metal rods arranged in parallel, linked electrically by a radio frequency (RF) voltage supply, which generates an alternating electrical field between the rods. Ions inside the quadrupole move

perpendicular to the field, in the *z* direction and start spinning within an imaginary cylinder created by the RF voltages. The diameter of the imaginary cylinder depends on the mass-to-charge ratio (m/z) of the ion and the RF voltage. Only ions within a certain m/z range are allowed to pass through the quadrupole. Mass analysis depends on the RF voltages and direct current (DC) applied. Mass accuracy in a quadrupole is in the order of hundreds of ppm., mass resolution depend on the ratio of the RF and DC voltage And the range of m/z can vary between 300 and 4000 [189].

- Quadrupole ion trap is a variant of quadrupole, but quadrupole-ion-trap has an electric field in three dimensions and deals with m/z range, mass accuracy and mass resolution similar to those showed by quadrupoles, but they can be increased by modifying the voltage scan rated [189]. A remarkable feature of the quadrupole ion-trap is the ability to carry out MS^n experiments, where multiple collision-induced dissociation can be executed consecutively.

- Time of flight (TOF). This technique separates the ions according to the velocity reached, in a TOF drift tube where a potential is fixed to accelerate the ions until they get to the detector. The TOF theory basically builds on the following equation that allows relating the mass of a ion over its charge (m/z) to the applied voltage (V), its time of flight (t) and the length of field free drift region (L):

$$m/z=2V(t/L)^2 \quad \text{Eq. 1}$$

According to equation X, the ions with lower m/z achieve higher velocities (around 100 km/s for a 100 m/z ion to 40 km/s for a 1000 m/z ion) and reach the detector first than those ions with higher m/z ratios. Then, knowing the time t that the ion takes to reach the detector, the applied voltage V and length of the TOF drift, m/z is deduced. Mass accuracy in TOF instruments is generally in the order of 5 to 25 ppm.

- Fourier-transform ion cyclotron resonance, FT-ICR or Fourier-transform Mass spectrophotometer FTMS uses a magnetic field to determine m/z of ions, but unlike sector instruments, the kinetic energy of the ions is about tens of electron volts, eV. Ions are trapped at the magnetic field and their cyclotron frequencies are measured and then converted to m/z values. This allows achieving very high resolutions and high mass accuracy inclusively below 1 ppm.[189]

Commercial instruments usually combined different principles. This is the case for tandem mass spectrometry, such as Quadrupole-Time Of Flight (Q-TOF). There are two stages of MS carried out. In the first, ions of a target m/z are isolated from the ion flow leaving the ionization source. The isolated ions can increase their internal energy, provoking fragmentation which can be analyzed in a second phase by TOF [189]. This process, usually called MS/MS or $MS^{2,}$ can be re-produced several times (MS^n) to help in the elucidation of the atomic structure the parent ions. Given the combination of the selectivity of Quadrupole and the high mass resolution, sensitivity and mass accuracy proper of TOF [193], this mass analyzer is suited for mass spectrometry targeted and untargeted analysis. In addition Q-TOF offers almost unlimited mass ranges, and fast scanning speeds. Consequently Q-TOF analyzers were the technique under which mass urine and broth cultures were analyzed in the present study.

3.2.2.2.3 Spectrometry Analysis Applied in this study.

For this study, the instrument used was a UPLC-PDA, coupled with Electrospray Ionization in tandem with a Quadrupole and Time-Of-Flight Mass analyzer (ESI-Q-TOF) (Waters Synapt G1, Waters Corp., Milford, MA, USA**) (Figure 3.8)**.

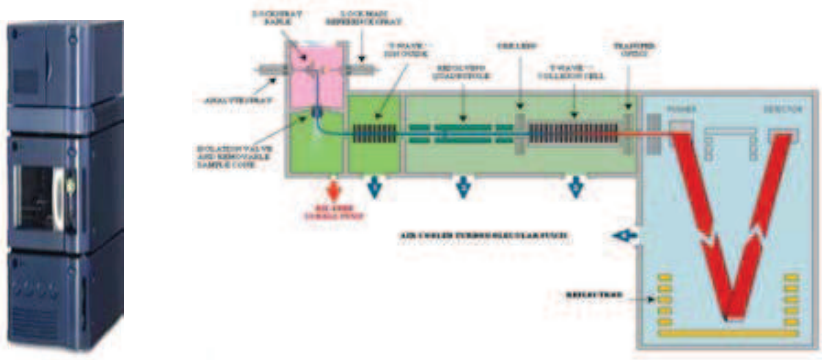


Figure 3.8 Illustration of a mass spectrometer MS V-Mode and each one of their parts, along with UPLC that become a powerful tool for metabolite analysis

Mass spectrometry data were collected within the range of 50 to 1000 m/z. Because chromatographic separation was hyphenated with mass spectrometry, the retention time and the time at which the ion reached the detector are relevant information that could be used for the elucidation of molecular compounds. To ensure repeatable results concerning retention time RT, signal intensity and mass accuracy, the instruments were calibrated at constant periods of time; and, strict cleaning procedures of the source and

other different parts were performed according to the main principle of Good Laboratory Practice including quality system of management controls

The ESI-Q-TOF/MS was operated in positive ion mode, with a desolvation gas flow to

330 L/hr at 350 °C of temperature. The cone gas set to 50 L/hr and the source temperature set to 120 °C. Capillary voltage was 2.3 kV. Mass scan (MS) mode and MS/MS fragments were measured from m/z 50 to 1000.

The scan time was 1 s and the interscan time 0.02 s. All analyses were acquired using leucine-enkephalin as the lock mass (*m*/*z* 556.2771) at a concentration of 0.125 μmol/L; and flow rate of 50 μL/min to ensure accuracy and reproducibility and also in a continuo mode. For MS/MS a collision energy ramp was 10 to 25 eV.

These spectrometry analyses supply different data, global information like the total ion current plot signal versus time (TIC) and specific ion chromatogram spectra for each scans. An example of a TIC and MS ion chromatogram spectra is shown on **Figure 3.9**.

3.2.2.2.4 Calculation of Accurate Mass and Error

The monoisotopic masses of compounds were calculated for [M+H] adducts with the following formula:

Measured mass [M] = Measured mass of adduct [M+H] + Electron- Proton

Eq. 2

With mass of proton= 1.007825035 amu and electron (e^-)= 0.00054857 amu

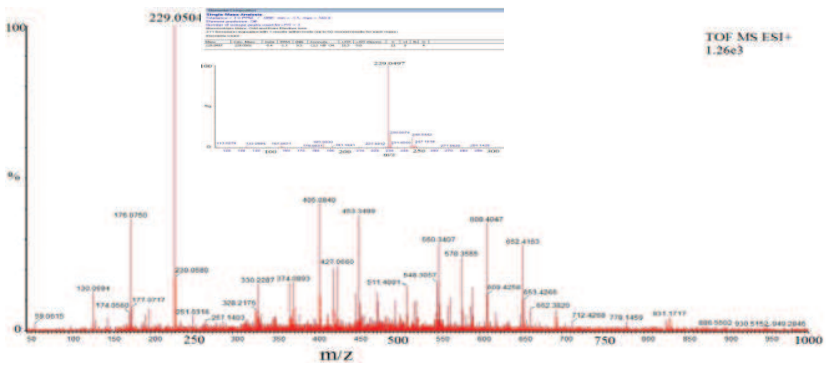
The error for a given atomic formula was expressed in ppm and calculated as:

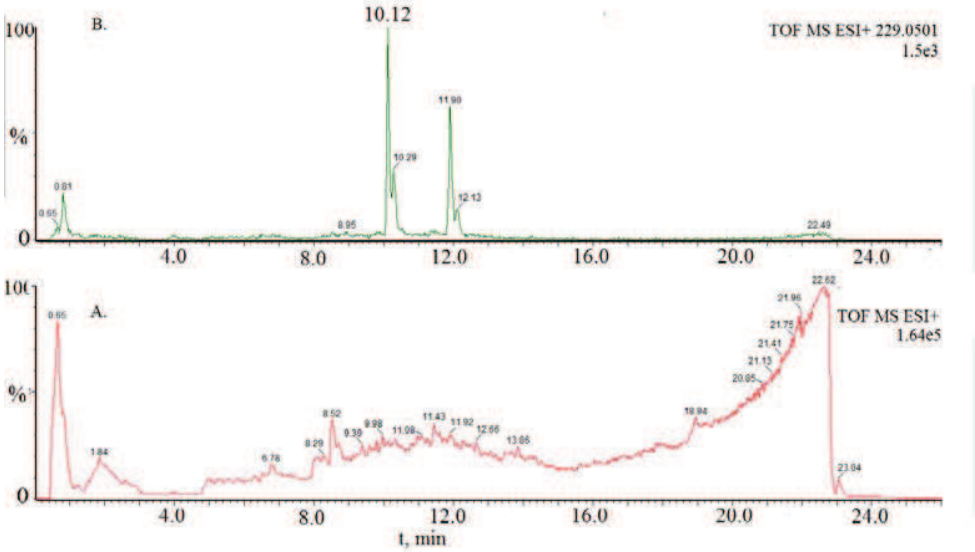
Error =10^6 x (measured mass – calculated mass)/ calculated mass

Eq. 3

With the calculated mass corresponding to an atomic formula calculated with:

Monoisotopic mass C=12.000000, H=1.007825035, O=15.99491463.





3.2.2.2.5 Identification and Annotation of Major ET-Metabolites by ESI-QTOF/MS

Except for the UA, UB and EA previously identified with the help of pure commercial standards, the other targeted metabolites were annotated according to various methods. Although we were able to determinate the exact mass of compounds with mass accuracy always below 5 ppm and often below 3 ppm, this result does not permit unambiguous identification. A single exact mass may correspond to various molecular formulas, which at their turn could correspond to thousands of isomers and tautomers. Some well-known rules [194] in spectrometric analysis allow reducing the number of formulas taking into account the isotopic abundance and patterns along with element number restrictions. Nonetheless, the reduction of candidates is not enough to allow identification based only on these results. Other clues must be added, such as retention time, UV spectra, MS^n pattern fragmentation to annotate the identity of ions in a more reliable way.

Glucuronide forms of UA and UB, corresponding to UAG and UBG, were unequivocally identified (1) by matching their UV spectra with the ones already

published in literature [58], (2) by matching their exact molecular weight (UAG (M-H+) m/z 405.0822 and UBG (M-H+) m/z 389.0873 respectively), (3) by matching UV spectra of UA and UB after treating urine samples with β-glucuronidase to remove the glucuronide moiety, (5) by matching the exact mass of UA and UB and observing a neutral loss of 176 Da corresponding to the loss of a glucuronide moiety when performing MS^2 at 15 V.

The urolithin UC, UD and their isomers, and UM5 were only annotated taking into account both, the important similarity of their UV spectra with those reported in literature [58], and to their observed exact mass. MS^2 fragmentation was also performed and similar patterns were observed, but as the fragmentation patterns were not available in data bases, structure elucidation was only tentative. In some cases also, the correspondence between order of elution and hydrophobicity estimated by Log D (Log P at physiological 7.4 pH) of proposed structure was taken into account.

Other ions, methylated and glucuronide conjugate of UA, UB, UC and UD, as well as precursors as HHDP were annotated with a lower probability considering only their exact mass and fragmentation pattern by MS^2.

The fragmentation pattern, neutral loss and molecular weight of fragments were analyzed and compared with similar compounds found in literature data and on-line libraries (ReSpect (http://spectra.psc.riken.jp/), Metlin: Scripps center for metabolomic (http://metlin.scripps.edu/index.php)), thus allowing a tentative assignment of the predicted structure with relatively high probability.

3.2.2.2.6 Specific handling of mass spectrometry data

A list of 25 targeted ETs metabolites was established and the search for finding the exact mass of adduct [M+H] was done in two ways.

First, manually by screening the MS spectra from all *in vivo* and *in vitro* samples making use of the MS ion extracted chromatogram (**Figure 3.9B, 3.9C**), accepting differences no larger than 3 ppm respect to the exact mass m/z^+. Secondly, by converting the raw data provided by the MassLynx software into a more manageable dataset, using the embedded software Databridge (MassLynx software). MS data acquired in continuous mode was first centered on the lock mass of leucine-enkephalin by using the MassLynx tools. This new data file was transformed by Databridge into

another file NetCDF to facilitate their treatment by MZmine [195]. After mass detection, chromatogram construction, deconvolution and alignments by MZmine software, a new file reporting the retention time, m/z^+ of ions and base peak intensity was generated and read in Excel. This file was used to profile the different samples with MS data. For reported compounds, mass of ions (m/z^+) were matched to exact mass reported considering a molecular weight tolerance inferior to 5 ppm in all cases.

3.2.3 CLINICAL STUDIES WITH HEALTHY VOLUNTEERS

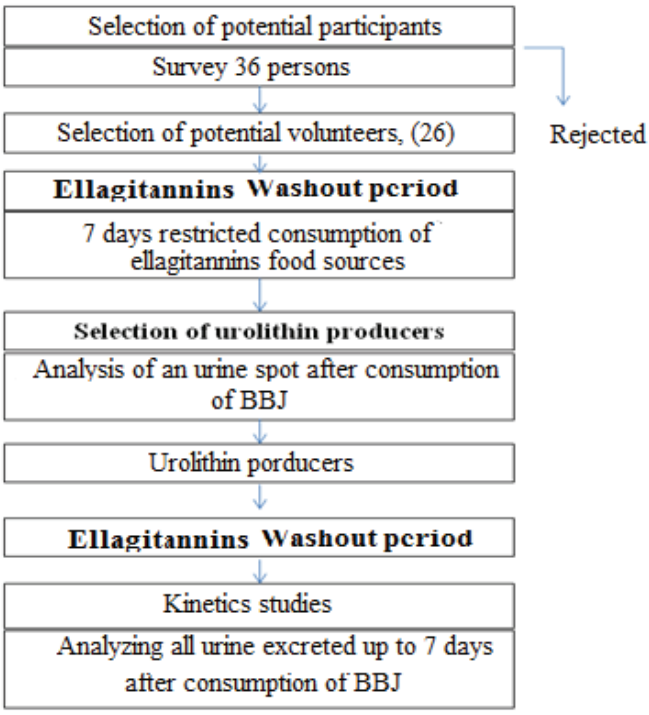
For the clinical studies with healthy volunteers, it was decided to follow up the excretion of ETs metabolites in urine. Urine is a key biological matrix in which has been identified more than 2651 metabolites species [196]. Urine, reflects metabolic disregulation, providing information about system-wide changes in response to physiological or disease processes [183]. Its collection is noninvasive and can be easily sampled in a serial manner, allowing temporal metabolic changes to be studied.

3.2.3.1 Study Design

The study complied with the Helsinki Declaration, and the University of Costa Rica Ethical Committee approved the protocol proposed, and the participants gave their written consent prior to participation. *In vivo* trials were divided in two steps, **Figure 3.10**: first at all, a preliminary step carried out to select urolithins excreters within a cohort of 26 volunteers chosen previously according to survey applied; and the second one, a kinetic study to approach to the metabolic pathway followed by ETs after volunteers drank a single dose of BBJ

3.2.3.1.1 Selection of volunteers

Ten men and sixteen women (non pregnant nor lactating), in good health were selected based on their medical history, (no gastrointestinal illness or chronic disease, nor participating in a special diet regimen, and had not been prescribed with antibiotics for at least 6 months before the study)



Once the volunteers were selected, and after they gave the consent to participate in the trial, they were asked to follow a free-polyphenol diet. Certain products, such as berries, pomegranate, nuts, grapes, chocolates wines were prohibited for seven days. After this polyphenols washout period, juice bags were thawed diluted 50:50 with water and added with sucrose until reach 12°Brix, and then each one of the 26 volunteers ingested a 250-mL of this diluted juice. About 46 to 56 hours after juice ingestion one single urine spot was taken and analyzed for urolithins A or B or their corresponding glucuronides. The diet restriction was kept until the day they provided the urine samples. **Figure 3.11** illustrates the time-steps of the study followed.

3.2.3.1.2 Kinetic studies

From the 26-volunteer group, nine volunteers were selected to follow the kinetic of ET-metabolism. The study was carried out applying the aforementioned methodology, but with some small modifications. After a 7 day washout period they were invited to provide a urine sample and then drink a 250-mL of the diluted BBJ. From this time and up to seven days, all the urine excreted was collected, homogenized, the time and volume were registered, and then a 10-ml aliquot was added with 0.1% (w:v) of

ascorbic acid and kept frozen at – 40°C for further analysis. For some volunteers, the kinetic study was repeated at different periods of the year. Between each intervention period, volunteers did not follow any type of diet restriction.

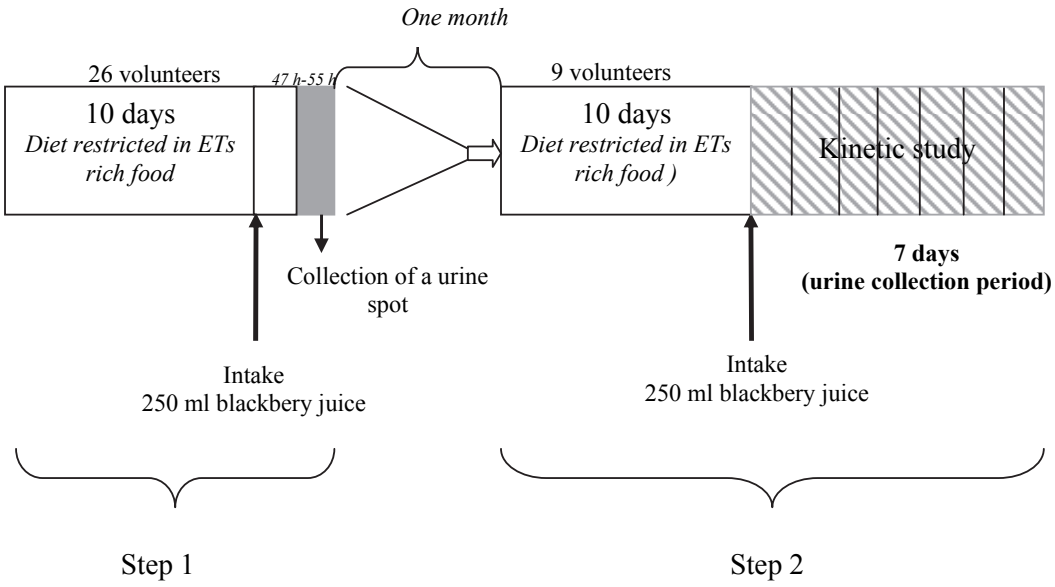


Figure 3.11 Layout of study design, different steps followed

3.2.4 IN-VITRO FERMENTATION WITH STOOLS

To disentangle the intricate metabolic pathway of polyphenols, first, we must understand how colonic microbiota converts these complex compounds into bioactive molecules, as well as to reveal how polyphenols may modify microbiota composition. *In vivo* human or animal intervention result appropriate physiological models to get a better understanding on the metabolism of polyphenols, since these type of models consider the interaction of colonic microbiota with the host system functionality such as immune or neuroendocrine system, gut absorptive process, or digestive tract secretions [16, 129]. However, practical and ethical reasons limit its applications [129]. *In vitro* models are low cost, easy to set up, they have no ethical restrictions, facilitate use of hazardous substances; and their major inconvenience is related to their limited interaction with the host system functionality [129]. *In vitro* models range from simple batch systems using fecal stools or defined mixed populations of bacteria until complex multistage continuous cultures that permit to carry out dynamic fermentation that simulates the different large intestine compartments and their functions. Fecal batch incubations have

been widely used to approach the gut microbial polyphenol metabolism, to characterize microbial fermentation products of dietary polyphenols, and also to confirm the high human interindividual variability in colonic microbiota composition that has been related to polyphenol bioconversion capacity [16].

3.2.4.1 Subjects

All 26 participants in the *in vivo* cohort donated a fecal sample between 0 and 48 hours after the ingestion of blackberry juice. These samples were divided in two parts; one to be used as starters in batch culture incubations to approach ETs metabolism by *in vitro* trials, and the other one to describe the bacteria involved in the ETs metabolism by independent culture techniques, **Figure 2.12.** Individuals that donated samples before 24 hours were asked to donate other fecal samples at 48 (FS48), 72(FS72), 96(FS96) and 120(FS120) hours after drinking the juice, in order to evaluate the effect of BBJ on the microbiota profile, and also to evaluate the influence on the urolithins production. All fecal samples were collected in sterile bottles and frozen immediately at -80°C until their use.

3.2.4.2 Starter preparation

An homogeneous suspension 1:9 (w:v) of thawed fecal samples in a 0.8% NaCl solution previously sterilized, was prepared in the Interscience stomacher (400ml Bagmixer-400W). Subsequently the suspension was filtered with a sterilized muslin cloth.

3.2.4.3 Growth medium

The growth medium consisted of brain heart infusion (BHI) medium (37 g/L), L-cysteine (0.5 g/L), and resazurin (1 mg/L), prepared in distilled water and adjusted to pH 7.4 with NaOH. Then it was sterilized at 121 °C for 20 min.[17].

3.2.4.4 Batch-culture incubation

All batch-culture vessels containing 10-mL of growth medium, 1 mL of starter and EA or BBJ dissolved in DMSO, were incubated at 37 °C in sealed jars (Oxoid Ltd, Basingstoke, Hampshire, England) under anaerobic conditions by using AnaeroGen sachets (Oxoid Ltd, Basingstoke, Hampshire, England). Only two controls were carried

out, flora in the basal medium without polyphenols and polyphenols in the basal medium but without starters.

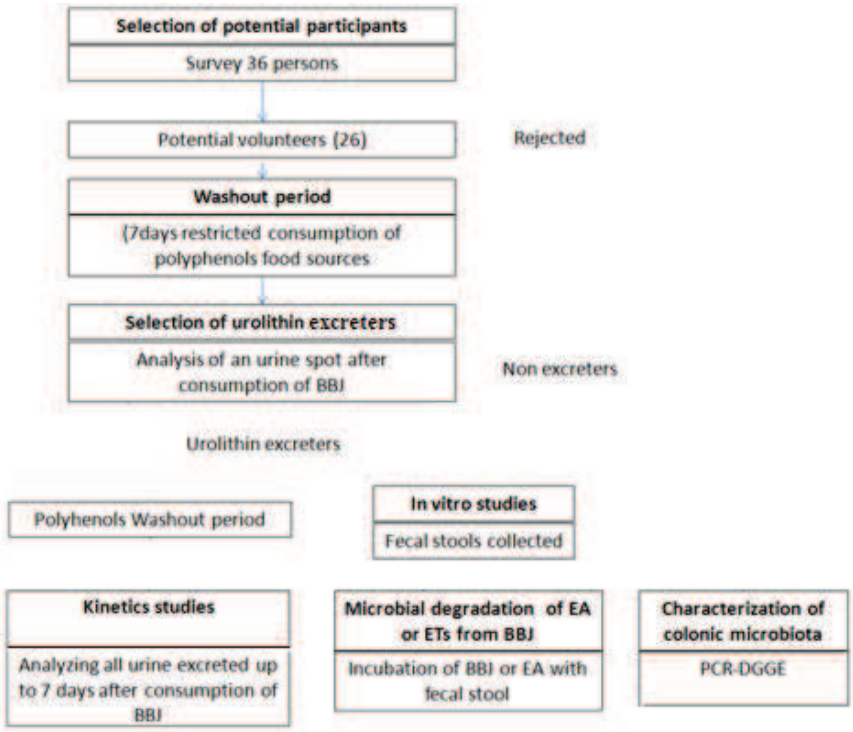


Figure 3.12 Scheme of *in vitro* test followed to identify microbial metabolites and potential microbial species involved in ETs degradation

Test 1. Degradation of ETs and EA by Colonic Microbiota

Eight fecal stools donated in the first 24 h after drinking the juice were used as starter for the batch culture vessels containing 0.14 μmol/ml EA equivalents from BBJ (diluted 1:1 with DMSO) or 0,05 μmoL of EA solution (0.3 mg/ml DMSO). All these batch culture vessels were incubated for 120 hours and their progress was followed by taking samples at 24, 72 and 120 h to be analyzed by LC-MS for ET-metabolites.

3.2.4.4.1 Test 2. Effect of BBJ ETs on the Colonic Microbiota Performance

Fecal stools donated at 48(FS48) and 96(FS96) hours, from one individual were used as starter to ferment EA and BBJ. ETs concentration for BBJ vessels was 0,18 μmol/mL EA equivalents and for EA vessels was 0,13 μmol/mL. Batch-culture vessels were incubated, as aforementioned, in sealed jars at 37 °C, for 120 hours. Batch-culture vessels contained 38-mL of growth medium and 10 mL BBJ (1:1 v/v DMSO) or 43 mL

of growth medium and 5 mL EA (0.4 mg/mL DMSO), inoculated with 2 mL of FS48 or F96 prepared as was described 3.2.4.4.

3.2.4.5 Metabolite recovery from fermentation broth

All samples were extracted based in the method previously described by Cerda, [17]. Briefly, each fermentation broth was extracted with diethyl ether (1:2 v/v mL x 3). The organic phases were pooled and evaporated under reduced pressure until dry, and redissolved in 1 mL of MeOH. All these methanolic fractions were filtrated through 0.45 μm filter and analyzed by LC-MS for ET-metabolites, and they were triplicated.

3.2.4.6 Metabolite Extraction from Feces

Fecal stools were extracted according to the method described by Cerda [17] and analyzed for ETs metabolites content. One gram of stool feces with 15 mL of MeOH:H2O:HCOOH (80:19.9:0.1, v:v) was homogenized with an Ultraturrax T25 (Henkel, Germany) 24000 rpm for 1 min and then filtered through sterilized cheesecloth. The filtrate was centrifuged for 7 min at 14000g (MiniSpin plus Eppendorf, Hamburg, Germany). The supernatant was filtered through a 0.45 μm filter, and analyzed by UPLC-DAD/ ESI-Q-TOF/MS.

3.2.4.7 Determination of Bioconversion Ability Index of Stools

An ability index for the bioconversion of ETs from blackberry juice to EA and Urolithins was computed. EA index was the percentage of bioconversion of ETs to EA after 72 h of anaerobic fermentation of blackberry juice with the different stool inoculums. Urolithin index was the sum of percentage of bioconversion of ETs into UM5, UD, UC and UA at 120 h of anaerobic fermentation of blackberry juice with the different stool inoculums.

3.2.5 PROFILING OF INTESTINAL MICROBIOTA

3.2.5.1 Culture Independent Techniques for Studying Anaerobic Microflora

Culture-dependant microbiological techniques are not well adapted for the identification of anaerobic flora from the gut; fortunately important advances in molecular techniques based on the study of DNA extracted directly from complex ecosystem have been achieved. Molecular techniques for rapid screening of complex microbial communities

have been developed on the last two decades, based on specific analysis (separation and /or sequencing) of ribosomal DNA (rDNA). Polymerase Chain Reaction amplification coupled to Denaturing gradient Gel Electrophoresis (PCR-DGGE) is a powerful molecular fingerprinting method that allows the screening of multiple samples and the study of the microbial communities dynamics in ecosystem, such as the intestinal microbial community[197-201].

3.2.5.1.1 Polymerase chain reaction (PCR) amplification.

Polymerase chain reaction amplification is probably the most simple, powerful (sensitive, specific, reliable) and fast method to duplicate DNA molecules. PCR is an *in vitro* cloning of specific DNA fragments by cycles of enzymatic DNA synthesis. PCR is carried out in some steps, as illustrated by **Figure 3.13**. 1) Denaturation of DNA template. 2) Annealing of primers; and 3) Extension of new DNA strands by a DNA polymerase and deoxyribunocleoside triphosphates (dNTPs). This process is repeated for 25 to 35 cycles to get sufficient material. **Table 3.2**., depicts the most common primer used in analyzing microbial communities.

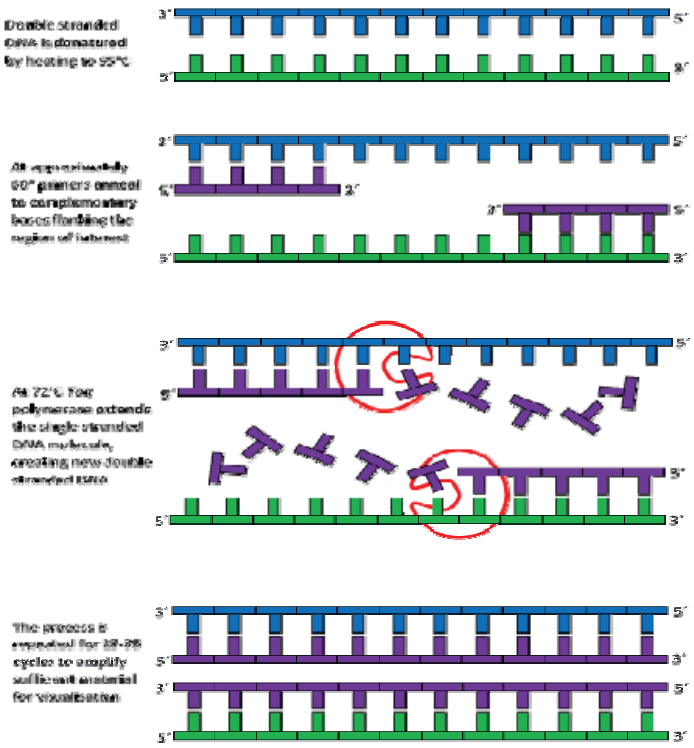


Figure 3.13 PCR process. 1. Denaturing, 2. Annealing, 3. Extension, 4. Cycles [202]

To maintain partial denaturation of the DNA fragment amplicons during DGGE migration, a GC Clamp (GC rich sequence of guanines G and cytosines C, 30-40 bp) is attached to the 5' end of one of the PCR primers. [198]

Table 3.2 PCR primers targeted for 16S rRNA gene, without GCClamp [198]

Primer set	Sequence (5'→3')	Amplicons length (bp)	Targeted hypervariable region
357fGC -518r	CCTACGGGAGGCAGCAG ATTACCGCGGCTGCTGG	194	V3
63fGC -518r	GCCTAACACATGCAAGTC ATTACCGCGGCTGCTGG	489	V1-V3
357fGC -907r	CCTACGGGAGGCAGCAG CCGTCAATTCCTTTGAGTTT	586	V3 -V5
F968GC -R1401	ATGGCTGTCGTCAGCT ACGGGCGGTGTGTAC	352	V8

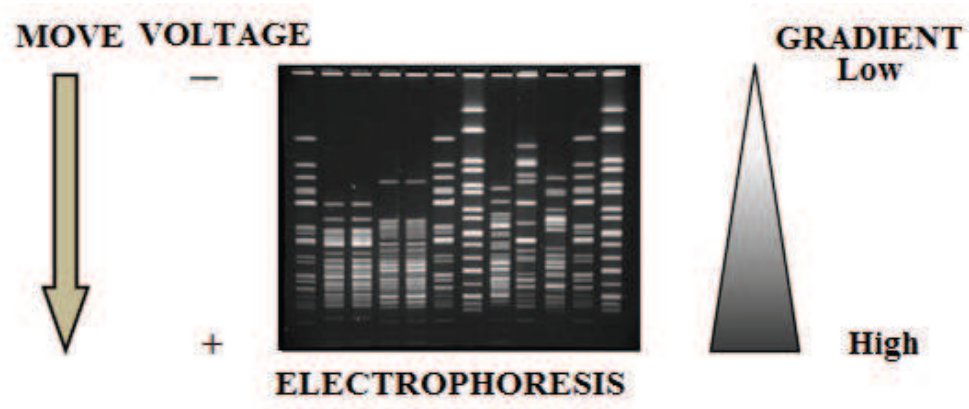
3.2.5.1.2 Denaturing Gradient Gel Electrophoresis (DGGE).

PCR-DGGE. It is fingerprinting approach is a rapid and efficient method widely used in microbial ecology to explore microbial diversity and dynamics in complex ecosystems such as gastrointestinal tracts. Its visual form to show results, allow a rapid approach to compare and monitoring changes in microbial communities [203].

DGGE is based on the separation of DNA fragments according to their sequence. It is used to separate DNA fragments of the same size but with different sequences in a polyacrylamide gel containing a linear gradient of denaturing agents (a mixture of urea and formamide); due to the decreased electrophoretic mobility of a partially melted DNA molecule compared to double stranded DNA molecule, **Figure 3.14**., depicts the basis of DGGE. Molecules with different sequences stop migrating at different positions in the gel.

Segments of DNA for all species of bacteria or fungi in the source sample are amplified by PCR (polymerase chain reaction). Despite the fact that these genes are highly conserved, variations within regions of these genes and between species are used to identify the different organisms. The amplified segments of DNA can be separated into individual species bands by using DGGE; then by sequence analysis of excised bands different organisms in the sample can be identified. In DGGE analysis, the generated banding profile is seen as an image of all major microorganisms in the population. An

individual discrete band refers to a unique ‘sequence type’ or phylotype since co-migrating bands generally corresponded to identical sequences [204]. PCR-DGGE electrophoresis is carried out at constant temperatures, typically between 55°C and 65°C [197, 198, 205]



Some of the drawbacks of using PCR-DGGE are related to PCR amplification. DNA may not be recovered from all genotypes or PCR amplification may be inaccurate. Amplification errors in PCR, can lead to formation of chimeric and heteroduplex molecules; and preferential amplification.

In DGGE electrophoresis the main limitation is the use of small DNA fragments (lower than 500pb) that could lead to low resolution of complex samples, and the use of specific or universal PCR primers for a particular group [197, 206]. In addition, the same specie could show diverse migration patterns due to the heterogeneity of its rRNA genes [207]. In general PCR-DGGE results are strongly influenced by the primer system and the gradient of the DGGE gel and the DNA staining method employed [199].

3.2.5.2 Fecal microflora profiling

DNA profiling of the V2-V3 region of the bacterial 16S rDNA by PCR-DGGE provides a snapshot of the dominant species present in the fecal microflora, excepting the detection of sulphate reducing bacteria [208]. Intestinal microbiota is specific in each

gastrointestinal compartment [1, 62, 132]. Although most of the knowledge about intestinal microbiota is derived from fecal samples, the dominant community in those samples represent barely the composition of parts of the sigmoid colon lumen [1, 126]. Nonetheless, the gut lumen of the ileum, is the only host site where relatively high concentrations of native polyphenols can be expected [15]. Also as dietary constituents are first encountered by the luminal microbiota *in vivo*, it is appropriate and convenient to use the faecal microbiota in the *in vitro* colon model [63]. In addition, obtaining colonic microbiota from fecal samples is a non-invasive method, which appears as pertinent from the ethical and practical point of view [63].

On the other hand besides bacteria, fecal samples contain fats, inorganic matter, protein and undigested foods, in sufficient amounts to provide nutrients for the microbiological population. Obligatory anaerobic bacteria *Bacteroides* spp., *Eubacterium* spp., *Bifidobacterium* spp., *clostridia, lactobacilli*, anaerobic *cocci* and *Fusobacterium* spp., and also facultative anaerobic such as *Escherichia coli*, *Enterococci* and *Streptococci* have been the most common bacterial species found in feces after batch culture incubations [1].

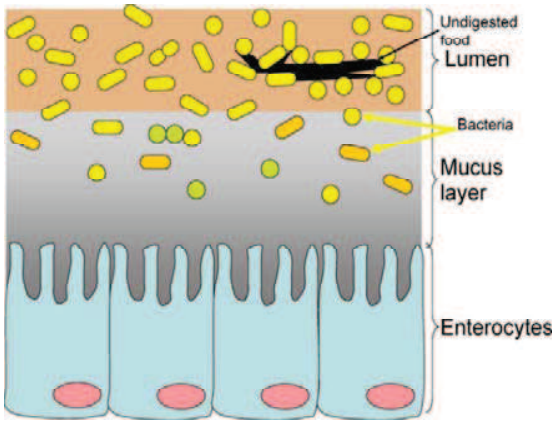


Figure 3.15 Spatial distribution of microbes at a selected site in the gastrointestinal tract [1]

3.2.5.3 Experimental Approach to Identify Bacterial species Involved in ETs Metabolism

To describe the bacterial species involved in ETs metabolism, a molecular approach of colonic microbiota from the 26 individual previously chosen was performed based on the sequence of the 16S rDNA gene. In addition, to evaluate whether ellagitannins from BBJ could exert any effect on the colonic microflora, fecal samples 48(FS48),

72(FS72), 96(FS96) and 120(FS120) were donated by four individuals, and were also analyzed by PCR-DGGE, for changes in microbiota composition and amount. DNA extracted from each sample was used as a template for amplification by PCR of a portion of the 16S rDNA gene, (the part of the DNA most commonly used for taxonomic purposes for bacteria), using universal primers. PCR DGGE profiles were then analyzed by multivariate analysis, PCA and PLSA in order to identify possible relationships between individual microflora diversity and the ability to release urolithins from blackberry ellagitannins. DNA bands of interest (corresponding to unique species) were cut from DGGE gels and sent to sequencing facilities for identification.

3.2.5.4 DNA Extraction

DNA was extracted following the protocol for isolation and DNA purification from stool samples, with the QIAamp DNA Stool Mini Kit" de QIAGEN (Venlo, Netherlands), according to the manufacturer manual. A frozen aliquot (180-200 mg) of each stool sample was utilized. The purity and concentration of the extracted DNA were measured using a Biospec-Nano spectrophotometer (Shimadzu Scientific Instruments, Japan). The integrity of genomic DNA was also visualized following electrophoresis migration through a 0.8% agarose gel.

3.2.5.5 PCR Amplification

PCR amplification of 16S rRNA genes. PCR amplification was carried out with the TopTaq polymerase kit from Qiagen (Venlo, Netherlands). The variable **V3** region from bacterial 16S rDNA was amplified by PCR with 338F (5'-CGCCCGCCGCGC GCGGCGGGCGGGGCGGGGGCACGGGGGGACTCCTACGGGAGGCAGCAG-3') and 518R (5′-ATTACCGCGGCTGCTGG-3′) primers (Sigma). The reaction mixture (50µl) was prepared according to the manufacturer and 5 µl of the above extracted DNA solutions was added. PCR amplifications were performed on a Biorad PTC100 thermal cycler. Reactions sequence were as follows: 3 min at 94 °C, 30 cycles of 30 s at 94°C, 30 s at 55°C, and 1 min at 72 °C, then 10 min at 72°C of final extension. PCR amplification products were checked by electrophoresis at 100 V for 30 min by running 5 µl of the PCR reaction on a 2% of agarose gel in TAE buffer. DNA was visualized after ethidium bromide staining on a UV transilluminator and photographs acquired with a coupled CCD camera and Gel Smart 7.3 software (Clara Vision, France).

3.2.5.6 Denaturing Gradient Gel Electrophoresis (DGGE)

PCR fragments were analyzed with Denaturing Gradient Gel Electrophoresis (DGGE) by using D-Code universal mutation detection system (Bio-Rad, USA), using the procedure first described by Muyzer [209] and improved by Leesing [210]. Samples containing similar amounts of PCR amplicons were loaded into 8% (w/v) polyacrylamide gels (acrylamide/N,N-methylene bisacrylamide, 37,5/1, Promega, France) in $1_{\times}$ TAE buffer (40 mM Tris–HCl, pH 7.4, 20 mM sodium acetate, 1.0 mM Na_2-EDTA). Electrophoreses were performed at 60°C, with denaturing gradient in the 30–60% range (100% corresponding to 7M urea and 40% v/v formamide, Promega, France). The gels were electrophoresed at 20 V for 10 min and then at 80 V for 12 h. After electrophoresis, the gels were stained for 30 min in ethidium bromide then rinsed with water for 15 minutes, visualized with a UV transilluminator and photographs acquired with a coupled CCD camera and Gel Smart 7.3 software (Clara Vision, France).

3.2.5.7 Analysis of PCR-DGGE gel profiles

The digitalized image of DGGE gels were analyzed using the Phoretix 1D software (v10.4, toollab, Ltd, Newcastle upon Tyne, UK). Briefly, the software analysis procedure consisted of 6 stages, 1) Lane detection, 2) Background noise subtraction, 3) Detection of the bands (spots) in each lane, 4) Calibration of the retardation factor (Rf), 5) Creation of a reference synthetic lane with all bands detected, and finally 6) matching the bands between the lanes. The Rf number records the position of the band in the lane, which is relative to the total length of the lane and is included between 0 and 1, with the start of the lane (inoculation spot) defined as 0 and the end of the lane defined as 1. Calibration of Rf was done considering the same reference lanes present in different gels, **Figure 3.16.** Then, for each corresponding Rf band, the relative band percentage for each lane was calculated by dividing the Gaussian volume of a band by the total volume of all the bands in the lane as:

$$\% \text{ band} = 100 \times (\text{Gaussian volume of the band} / \text{total volume of all bands in the lane}) \quad \text{Eq. 4}$$

The volume considers the combined area of pixels with color intensity above the noise threshold along with the grey scale intensity of each pixel. This measure was found useful to compare across lanes and gels where the loading or light exposition may have varied slightly. Deconvolution is also particularly useful in cases where two or more bands overlap. Profile deconvolution uses a curve-fitting algorithm of Gaussian curves. In order to facilitate the comparison between lanes from the same DGGE gel and band profile in different DGGE gels, two reference patterns (*Lactobacillus Plantarum* and *E.Coli*), and also common lanes in different DGGEs were used to homogenize the DGGE images.

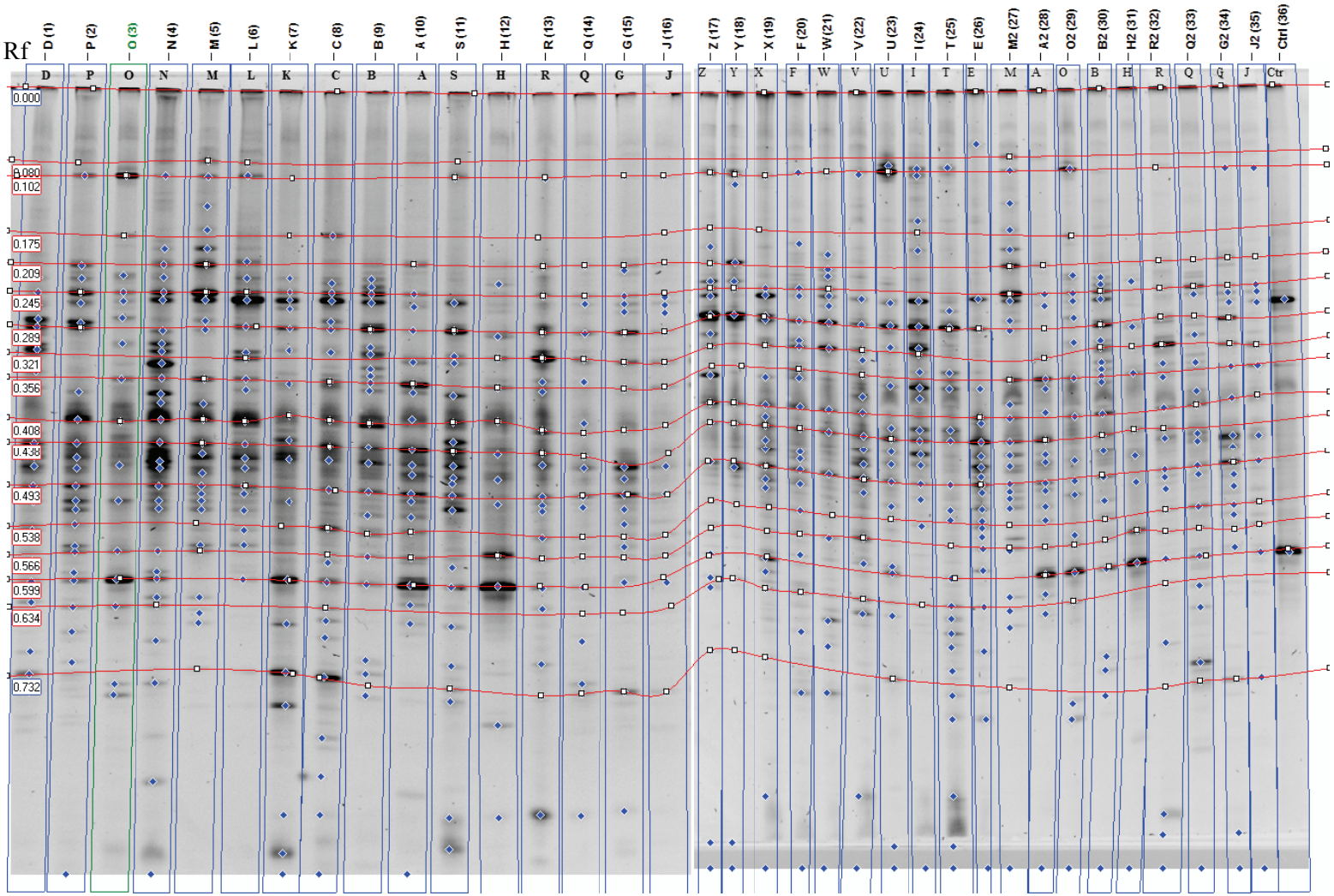
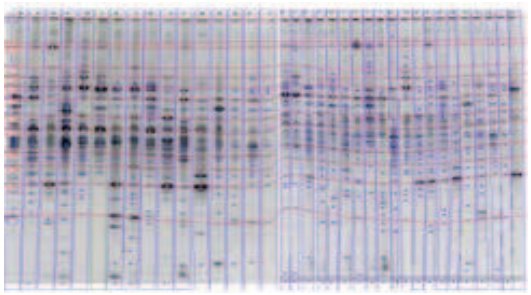


Figure 3.16 Example of the calibration of the retardation factor (Rf) between lanes and two different gels

Band position and in some cases intensity were the parameters used to facilitate their comparison and analysis. The Phoretix 1D software provides a table with different information, among them Rf, % band, peak height among others, than can be transferred as EXCEL file, as is illustrated by **Figure 3.17.**

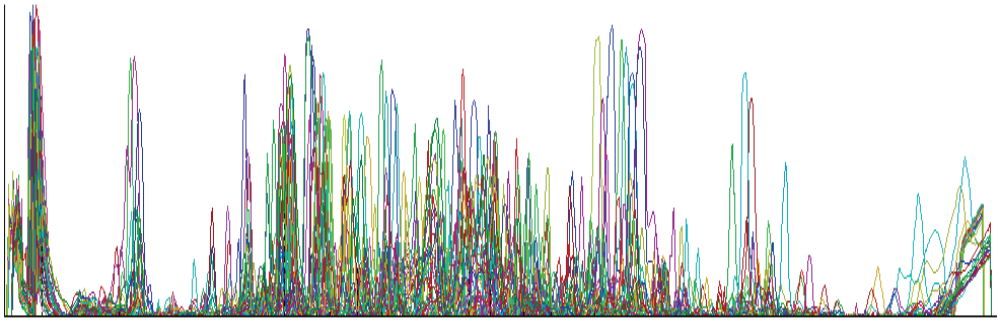


1) Lane detection

2) Background noise subtraction

3) Band detection

4) Calibration of the retardation factor (Rf)



Volunteers	Urolithin ind	RF 0.234	RF 0.238	RF 0.252	RF 0.262	RF 0.269	RF 0.294	RF 0.301	RF 0.315	RF 0.324	RF 0.328
A	37.15	0	0	1.441	0	1.779	1.013	0	0	0	0
A2	37.15	0	0	0.44	0	0.171	0	0	0	0	0.438
B	34.17	0.848	2.585	5.892	5.589	0	10.219	0	0.872	0	5.067
B2	34.17	1.284	3.13	5.08	4.795	0	12.168	0	1.073	4.983	0
C	28.95	0.278	0.8	2.834	0	0	1.591	0	0	1.652	0
D	5.37	1.103	0	0.626	0	1.952	5.228	8.576	0	2.422	8.561
E	2.77	0	0	0	9.855	0	5.527	0.319	0	0	
F	9.94	0.374	0	2.597	1.119	0	5.26	0	0	3.072	4.601
G	0.35	0	0	1.186	1.97	0.735	8.163	0	0	0	0
G2	0.35	0	0	1.884	1.973	0.962	8.036	0	0	0	0
H	0.28	0	0.391	6.092	0	0	0	3.972	0	0	2.454
H2	0.28	0	0.905	0	0	0		3.47	0	0	1.12
I	0.41	0	0	0	8.069	0	10.563	0	0	11.596	4.365

Figure 3.17 Process followed by Phoretix 1D® to obtain aligned bands between lanes and gels and Excel file with band % corresponding to each retardation time.

3.2.5.8 Recovery of bands from DGGE gels and sequence analysis

DNA bands were excised with a sterile surgical blade and transferred to a 1.5 ml microcentrifuge tube containing 100µl TE buffer and incubated at 4°C overnight. Subsequent purification steps were performed with Wizard® PCR Preps DNA Purification System (Promega), following the methodology described in this purification system kit.

3.2.5.9 Sequencing of excised bands

Sequencing is the process of determining the nucleotide order of a DNA fragment. Sequencing of purified DNA from DGGE bands was performed by GATC Biotech company (Germany) on ABI capillary sequencer system. DNA sequence analyses were processed using Sequence Scanner 1.0 (ABI) and resulting DNA sequences were compared with database sequences of the National Centre for Biotechnology Information (NCBI) as well as RDP (Ribosomal Database project). Briefly, the different steps followed are explained below:

Step 1: Interpretation of sequencing. GATC BIOTECH provides the nucleotide sequence of each one of the DNA fragments from each of the two DNA strands, so-called forward (F) and reverse (R). The encoding **Table 3.3** from IUPAC (International Union of Pure Applied Chemistry) shows the letter assigned to each base and also letters when ambiguities are present.

Table 3.3 Encoding for the four bases and for ambiguous positions in DNA sequences

Code	Meaning	Etymology	Complement
A	A	Adenosine	T
T/U	T	Thyamidine/Uridine	A
G	G	Guanine	C
C	C	Cytidine	G
K	G or T	Keto	M
M	A or C	Amino	K
R	A or G	Purine	Y
Y	C or T	Pyrimidine	R
S	C or G	Strong	S
W	A or T	Weak	W
B	C or G or T	Not A (B comes after A)	V
V	A or C or G	Not G (H comes after G)	B
H	A or C or T	Not C (D comes after C)	D
D	A or G or T	any	H
X/N	G or A or T or C		N
.	Not G or A or T or C		
-	Gap of intermediate length		

According with these reported codes, the quality of the sequence is analyzed, looking for ambiguities to correct them. **Figure 3.18** depicts an example of a sequence fragment in which there are two ambiguities in position 131 and 138. This process is carried out with both forward and reverse sequences.

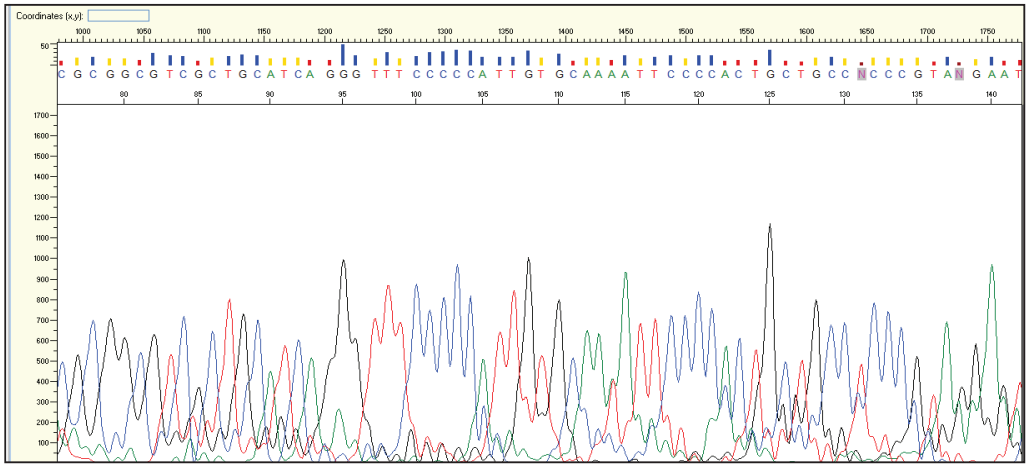


Figure 3.18 Example of DNA sequences provided by GATC BIOTECH

Step 2: Building of "Enter Query Sequence". When both DNA forward and reverse sequences are workable, the common sequence is identified and the complete sequences is reformed (using 5' and 3' end portions) "Enter Query Sequence".

Step 3. Query of databases. The "Enter Query Sequence" is sent on-line to a database (BLAST from National Center for Biotechnology information (NCBI)) to determine the homology with sequences already registered. A "Nucleotide collection" and the 16S ribosomal_RNA_Bacteria_and_Archaea database were used.

Step 4. Analysis of matching results. The BLAST results are analyzed considering the best match score with known sequences. Parameters such as maximum score, total score, query cover, E value and % of identity provided by the software are analyzed in order to annotate with the highest probability possible the identity of the bacterial species.

3.2.6 MULTIVARIATE ANALYSIS OF DGGE PROFILES

In order to compress the data obtained and try to evidence how microbiota profile may influence bioconversion ability into urolithins, multivariate analyses were performed.

Data matrixes were processed by Principal Component Analysis (PCA), Hierarchical Analysis (HA) and Partial Least Squares Analysis (PLS).

3.2.6.1 Principal Component Analysis (PCA)

Principal Component Analysis, PCA is a method usually applied to display data in a more simple way, and for exploratory analysis purposes. PCA reveals the internal structure of a data set in a way which best explains the variance in the data [211]. PCA is used to project multivariate data to a low-dimensional plot that assembles the major variations in the data into few axes, thus systematic variation is captured in a model that can cluster the samples as similar or dissimilar to each other [211, 212]. PCA can identify gross variability and find a direction of "maximum gross variability" that is usually consistent with group separation but does not discern between "among-groups" and "within groups" variability. Although PCA has an ample application in several fields, concerning analysis of DGGE gels, PCA treat the intensity of each band in DGGE gels as independent from the other bands; which can lead to wrong interpretation of the DGGE gel [207]. PCA analysis was performed using SIMCA software Version 13.0.2 (Umetrics, Sweden).

3.2.6.2 Hierarchical Cluster analysis (HCA)

Hierarchical Cluster Analysis (HA) of gels was performed with Phoretix 1D. Similarities between the band patterns from the different PCR-DGGE gels were computed using the Pearson correlation coefficient. Similarities or dissimilarities were displayed graphically as a dendogram. The clustering algorithms used to calculate the dendrograms was an unweighted pair group method with arithmetic averages (UPGMA) [213].

3.2.6.3 Partial Least Squares Analysis (PLS) and variants PLS-DA and OPLS

PLS is a statistical tool applied when trying to establish relations between clusters of observed variables X and responses Y. Similar to PCA, PLS methods projects original data onto a reduced space called latent variables [214]. PLS try to relate two data matrixes, X and Y, to each other by a linear multivariate model. A variant of PLS for discrete response data is the Partial Least Square Discriminant Analysis (PLS-DA) which focuses on class separation.

The orthogonal PLS (OPLS) model is another extension of the PLS model. It separates the systematic variation of variable matrixes into two parts, one that is linearly related (and therefore predictive) to response matrix Y and one that is orthogonal to Y. The X/Y predictive variation is modeled by the predictive components. The variation in X, which is orthogonal to Y, is modeled by the orthogonal components. OPLS is convenient for the treatment of noisy data. For both PLS-DA and OPLS, the interpretation of scores and loadings and other model parameters is analogous to that of a PLS model. PLS, PLS-DA and OPLS were performed also with SIMCA software.

To assess the validity of models, multivariate modeling was implemented using leave-sample-out cross-validation throughout. Parametric statistical tests such as the cumulative percent of the variation of Y explained by the model R^2Y_{cum}, predicted variation Q^2 and P-value of the ANOVA of the cross validated residuals of Y-variable were calculated by the same software. Variable importance for the projection (VIP) was calculated to reflect the importance of terms in the model both with respect to Y, i.e. correlation to all the responses, and with respect to X (the projection). The VIP plot is normalized, the average squared VIP value is 1.Thus, terms in the model with a VIP > 1 are the most important. Regression coefficients were also calculated and displayed with a confidence interval computed by jack-knifing.

4 METABOLIC FATE OF ELLAGITANNIN'S COLONIC DERIVATIVES

4.1 IDENTIFICATION AND QUANTIFICATION OF ELLAGITANNINS IN BLACKBERRY JUICE

ETs form blackberry juice were previously studied by our team and the two main ETs Lambertianin C and Sanguiin H-6 were identified by UV spectra, exact mass (2804.23 and 1870.16 respectively) and MS^2 fragmentation pattern [36]. The same procedure as previously published was followed for the identification and quantification of ellagitannins. **Table 4.1** shows the content of ETs and free EA derivatives of the juice that was used in the different trials. The ETs content is in accordance with previous studies on tropical highland blackberry (*Rubus adenotrichos*). The Lambertianin C and Sanguiin H-6 are the most important ellagitannins present in the juice that was given to the volunteers.

Table 4.1 Contents of ellagitannins in blackberry (*Rubus adenotrichos*) juice

Ellagitannins *(*mg EA equ/L*)*	
✓ Lambertianin C	298 ± 5[a]
✓ Sanguiin H-6	246 ± 5 [a]
Ellagic acid derivatives *(*mg EA equ/L*)*	8± 1 [a]
Total (mg EA equ. L-1)	552± 10
Total (µmol EA equ. L^{-1})	1822 ± 20

4.2 IDENTIFICATION OR ANNOTATION OF ETS METABOLITES PRESENT IN URINE AFTER INGESTION OF BBJ

A total of 26 healthy volunteers were chosen to perform a study on urinary excretion of ETs metabolites. In this case, a spot of urine was collected 51±5 h after ingestion of the juice. The UPLC-MS analysis of urine from volunteers allowed the identification of different ETs metabolites, principally conjugated forms of urolithins.

4.2.1 IDENTIFICATION OF UAG AND UBG IN URINES

After ingestion of tropical highland blackberry juice, the main ellagitannins derivatives found in human urine were urolithin A glucuronide (UAG) and urolithin B glucuronide (UBG). Both metabolites were identified by their UV spectra (**Figure 4.1**), and by their molecular weight (UAG (M-H+) m/z 405.0822 and UBG (M-H+) m/z 389.0873 respectively). They were confirmed by enzymatic treatment of urine samples with β-glucuronidase and also after running the MS^2. In both cases their respective aglycones, UA and UB, were identified, **Figure 4.1**.

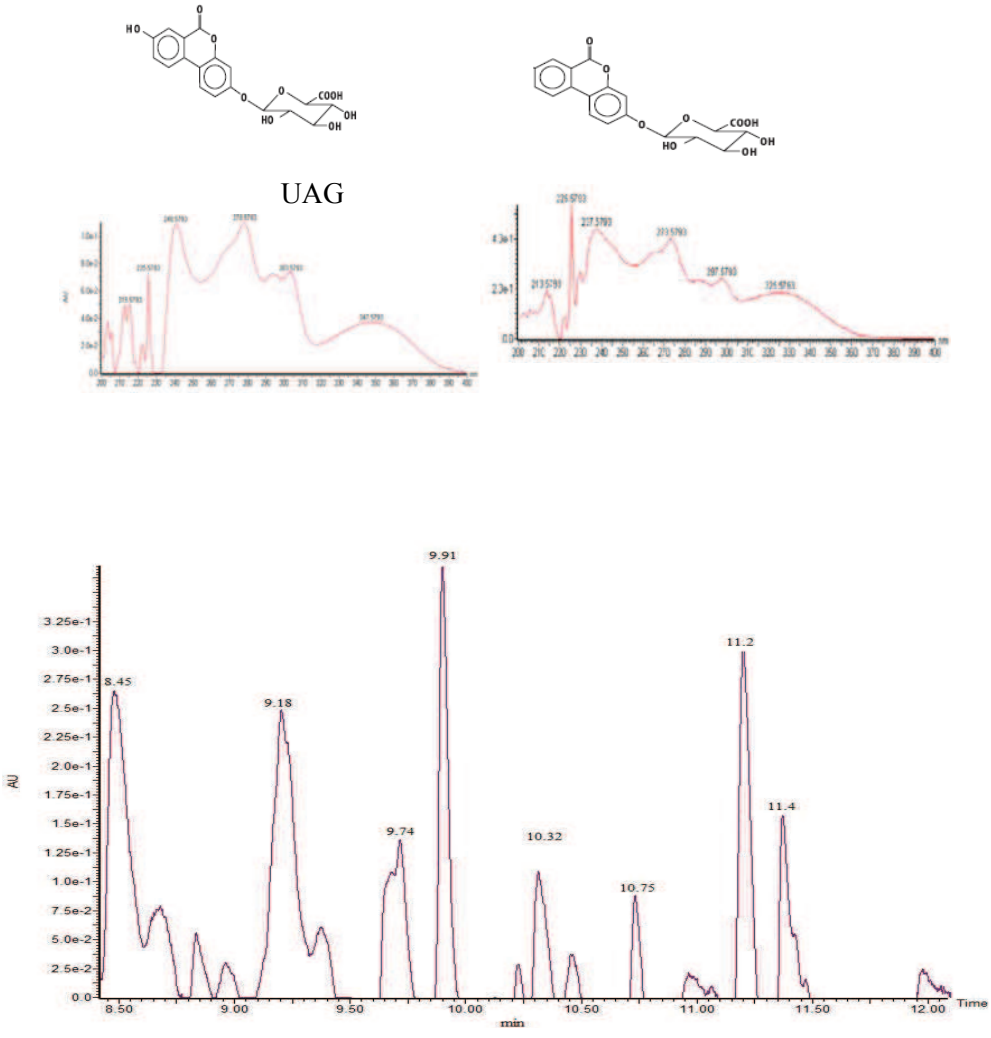


Figure 4.1 Example of UV chromatogram (at 278 nm) of a urine sample from a volunteer collected 3 days after ingestion of blackberry juice, including peaks and UV spectra of glucuronide forms of urolithin A (UAG) and B (UBG).

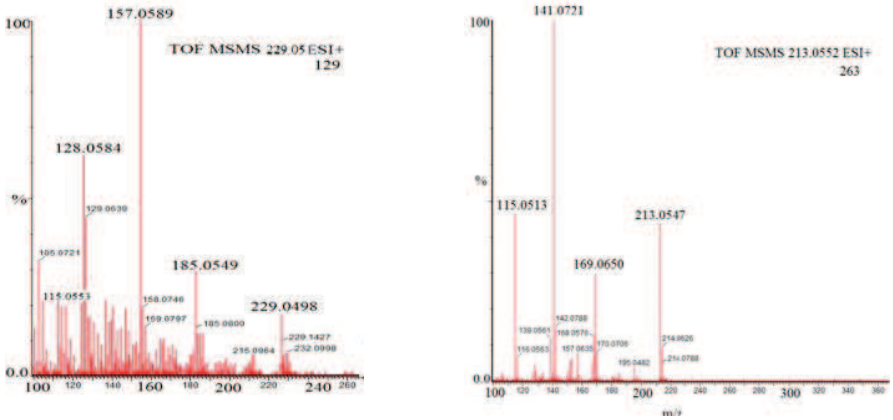
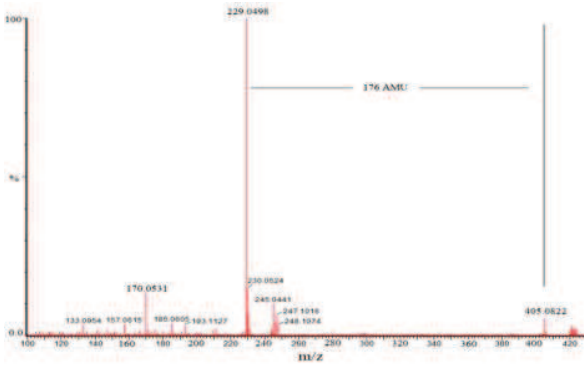


Figure 4.2 MS^2 Fragmentation of urolithin A and urolithin B

In MS^2 their corresponding fragment at (M-H+) 229 m/z and (M-H+) 213 m/z respectively along with a neutral loss of 176 Da (**Figure 4.3**) corresponding to the loss of a glucuronide moiety confirmed the presence of UAG and UBG as the major ETs metabolites. This result is not surprising as glucuronidation is the most important reaction in phase II metabolism [73, 215-220]. The conjugation of UA and UB aglycones occurs in the liver or intestinal cells under the action of UGTs UDP-glucuronosyltransferases in order to increase solubility and facilitate excretion through the urine [73]



4.2.2 ANNOTATION OF OTHER ETS METABOLITES FOUND IN URINE

High-resolution mass spectrometry analysis of urine was performed to annotate other compounds. The UPLC / ESI(+)-Q-TOF/MS and MS^2 allowed the tentative assignment of peaks to 14 other ET metabolites. In **Table 4.2**, these compounds are presented with their respective retention time, theoretical molecular mass $(M-H)^+$ for the ion, MS^2 spectral fragments in the positive mode (ESI(+)), assignment and the acronym used in this paper. **Table 4.2** shows that the small fragment (m/z 115.05) is common to almost all urolithins, except for UD and UM5 (m/z 114.05). This MS^2 fragment ion could be characteristic of urolithin compounds in a positive mode (ESI^+), although this fragment is also reported in the MS^2 patterns of some flavonoids. **Figure 4.4**., shows an example of a fragmentation pattern for UC and DMUC**,** as well as a tentative prediction of some of the fragment's structures.

Table 4.2 UPLC-DAD/ESI(+)-Q-TOF/MS and MS2 analyses of ellagitannins colonic metabolites found in urines of volunteers

Rt (min)	UV spectra λmax (nm)	MS $(M+H)^+$	MS^2 m/z *(% base peak)*	Assignment	Acronym
11,4	248,280,305,366	229,0501	185*(30)*,157*(100)*, 128*(65),115(20)*	Urolithin A	UA
13,3	249,276,301,333	213,0552	169*(32)*,141*(100)*,115*(47)*	Urolithin B	UB
10.1	255,304,349	245,045	170*(100)*,142*(15)*,115*(22)*	Urolithin C	UC
9,09	259,sh291,380	261,0399	218(*65)*,172*(85)* , 120*(87)*,114*(100)*	Urolithin D	UD
8.3	281,sh292,352	277,0348	260(*60*),232(*32*)188(45),114(12)	Urolithin M5	UM5
12.9		243,0657	229(43),203(65),170(33)151(100), 115(20)	Methyl urolithin A	MUA
9.3		273,0763	229(30),203(100),189(85),169(35), 137(45),115(70)	Dimethyl-urolithin C	DMUC
9,9	240,278,303,347	405,0822	229*(100)* 170*(10)*	Urolithin A glucuronide	UAG
11.3	237,273,297,325, 287,297,326	389,0873	213(100)	Urolithin B glucuronide	UBG
8.7		421,0771	245(100)	Urolithin C glucuronide	UCG
14.5		437,0772	261(100)	Urolithin D glucuronide	UDG
10,4	253, 365	303,0141	177(90),153(100),145(35)131(17)	Ellagic acid	EA
13,4		507,0775	331(100)	Dimethyl ellagic acid glucurionide	DMEAG
10.6		331,0454	165(25), 147(100),132(50),115(32)	Ellagic acid dimethyl ether	DMEA
9,1		305,0297	229(20),180(35),177(55),153(42), 131(100),103(35)	Hexahydroxydiphenyl	HHDP

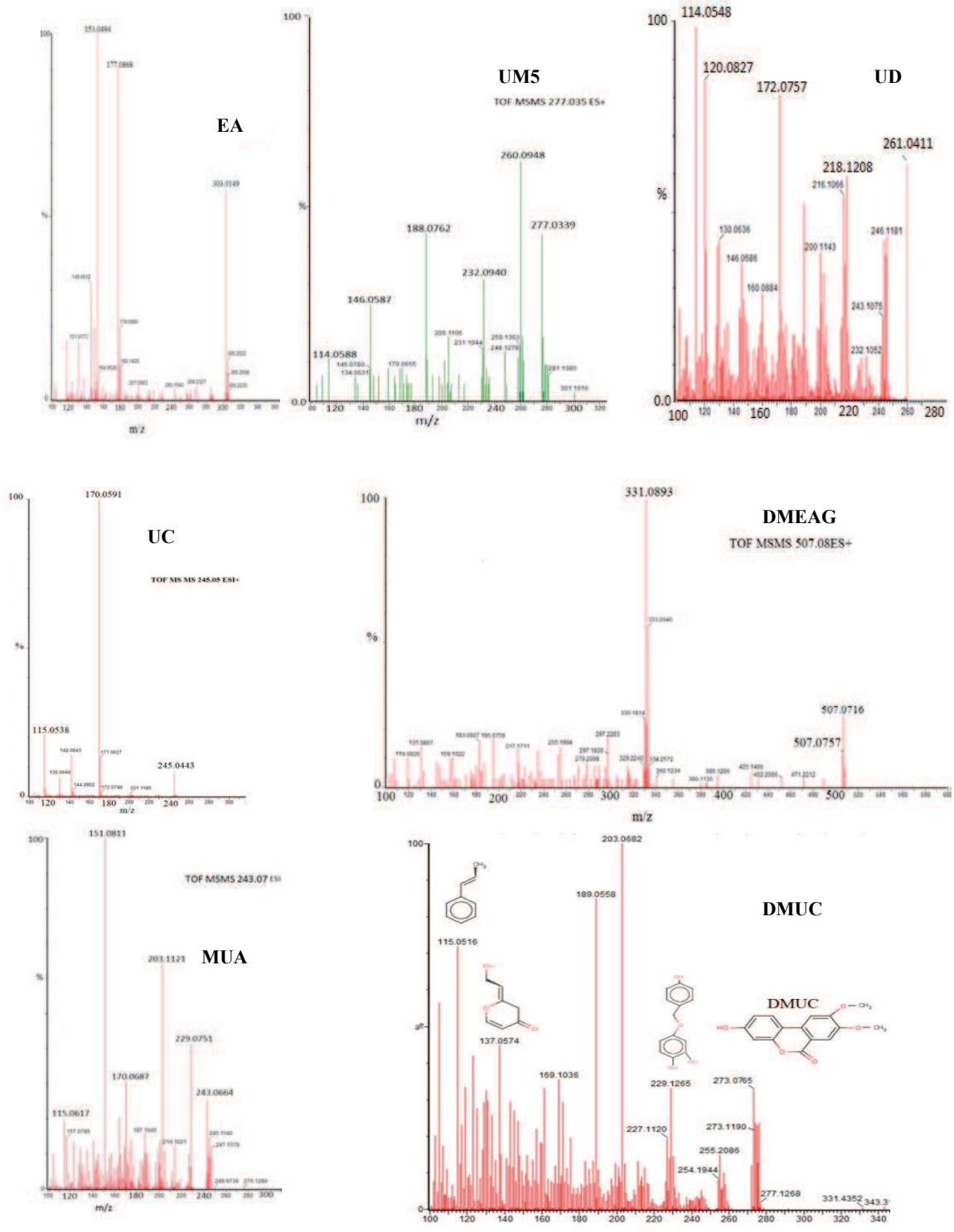


Figure 4.4 Example of MS2 fragmentation pattern of Ellagic Acid, urolithins: UM5, UD UC, dimethyl elagic acid glucuronide (DMEAG), methyl urolithin A (MUA) and dimethyl-urolithin C (DMUC), and tentative prediction of some fragment ions

4.3 DIVERSITY OF URINARY EXCRETION PATTERNS IN HUMANS

The concentration of main UA and UB derivatives (aglycone and glucuronide forms) detected in the spot of urine collected from the 26 participants, at 51±5 h after they ingested one shot of 250 mL of diluted blackberry juice is illustrated by **Figure 4.5**. To encode the volunteers, a letter was assigned to each one, but in this figure only the gender was used to present the concentration of UA and UB reached for each of them. The important inter-individual variability in the excretion of UA and UB derivatives was evident and in accordance with previous studies [43, 50, 221].

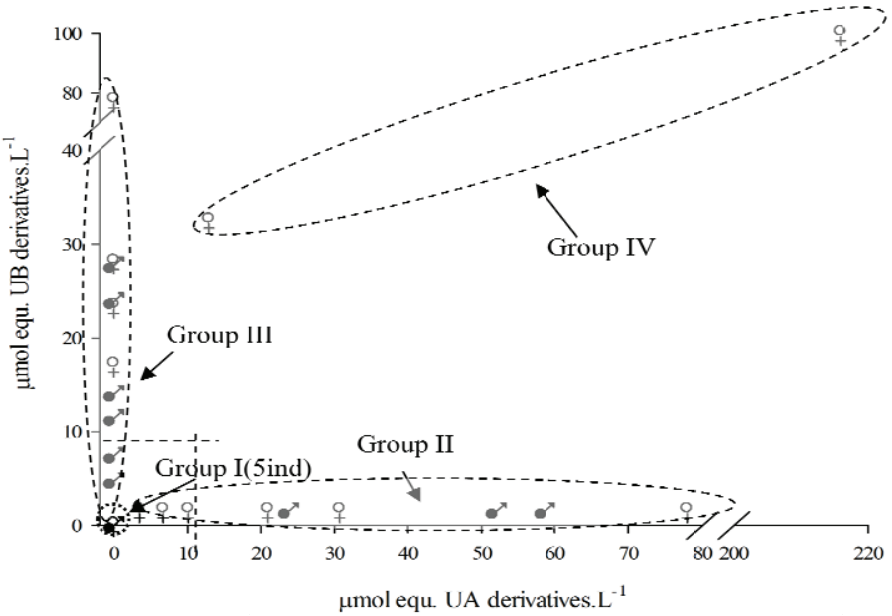


Figure 4.5 Concentration of primary urolithin A and B derivatives (aglycone and glucuronides forms) in a urine spot, from 26 volunteers, collected at 51±5 h after ingestion of 250 mL of blackberry juice diluted (50:50) with water**.**

This spot assay showed clearly two main groups, the first one comprised by 16 individuals (61%), who achieved urolithin concentrations > 10 µmol/L of UA/UB derivatives , thus categorized as high urolithin excreters; whereas the other 10 individuals (39%) did not reach important amounts of urolithins, considered as no or low urolithin excreters. To be more specific, people were clustered into 4 categories according not only the amount of

urolithins released but also by type of urolithins. A first group of 5 volunteers (19%) were labeled as non urolithins excreters at all, since they did not release UA or UB derivatives; a second group of 10 individuals (38%) where UA derivatives are largely predominant; a third group with 9 individuals (34%) where UB derivatives are largely predominant; and finally a fourth group with 2 individuals (7%) with a balanced concentration of UA and UB derivatives in their urine spot. Neither gender nor diet effect could be evidenced in the urolithins production.

As this trial was based only in the analysis of a simple spot, it was not possible to draw a decisive conclusion. However as this preliminary test shed lights on the degradation patterns of ETs, a second assay was set up to confirm those interesting findings observed. In the second assay all the urine excreted by nine participants that consumed a shot of BBJ was collected up to seven days after the juice ingestion.

4.3.1 INTER-INDIVIDUAL VARIABILITY OF URINARY EXCRETION OF UROLITHIN OVER A 4-DAY PERIOD

To assess whether the classification proposed concerning the ability to release urolithins after the analysis on a single urine spot is kept when a more extended monitoring is performed, a new study with 9 individuals from the initial cohort was carried out. They included volunteers from each one of the groups established; and the urolithins excreted was monitored up to the fourth day after the ingestion of BBJ. **Figure 4.6** depicts, the total percentage of conversion of ETs into UA and UB derivatives achieved for each one of the nine volunteers four days after ingesting a shot of 250-mL of BBJ. This intervention trial was repeated with four of the volunteers at different times over a year, to express the conversion reached in each intervention for the same individual, a number was added to their assigned letter. They are numbered in chronological order. Three groups corresponding to “no or low urolithin excreter” (individual D), “predominant UA derivatives excreters" (individuals F, G, O, R, and W) and "predominant UB derivatives excreters" (individuals C, H, and I) can clearly be observed. Although individuals F, H and O have the ability to release both UA and UB, it is evident that some of these metabolites

prevail over others, for which they were classified into the group prevalent. The total conversion of ETs into primary UA or UB derivatives excreted varies between almost 0% up to 37%, which could be considered as an ample variation. However, the conversion is even low, and there is no explanation for the fate of the other ETs. It has been proposed that other ETs could suffer an extensive degradations that produce smaller compounds such as CO_2 [49].

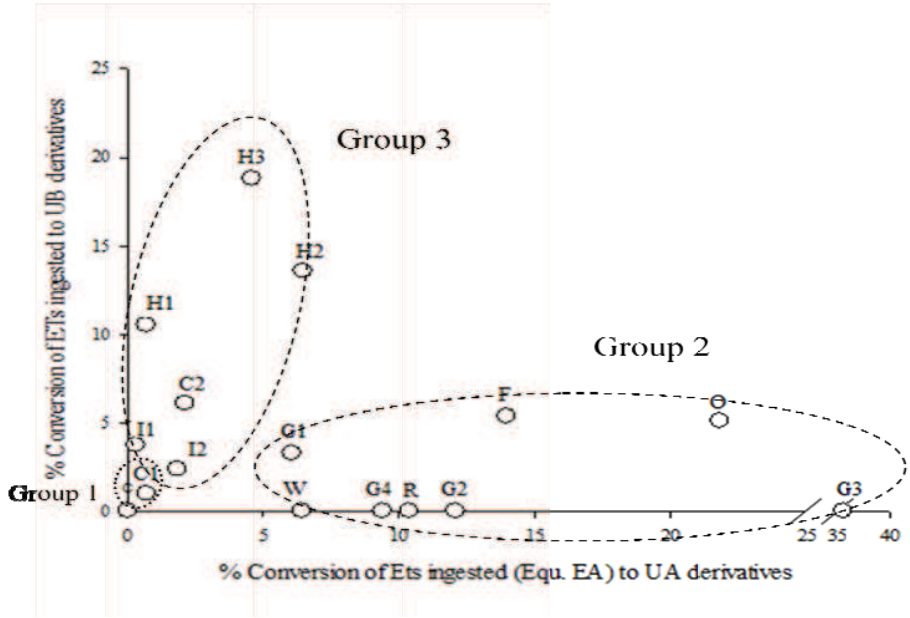


Figure 4.6 Total conversion of ETs to UA and UB derivatives (aglycone and glucuronide determined by UPLC/DAD) excreted in urine over 4 days after ingestion of 250-mL of tropical blackberry juice for 9 individuals (C,D,F,G,H,I,O,R,W) at different interventions.

This assay confirms the pattern discrimination of the ETs carried out from the previous assay, where only a urine spot analysis was carried out. Results obtained from that trial are in complete accordance with those achieved after four days of monitoring all the urolithins excreted in urine.

According to these results, at least three different patterns for the urinary excretion of main ET metabolites are observed in the population sample: individuals that release urolithins in non quantifiable amounts and then can be considered as non urolithin excreters; individuals that excrete predominantly UA derivatives and the third group those who release mostly

UB derivatives. This status appeared to be constant over time. According to the conversion achieved for individuals that participated several times in this assay, (C,G,H, I,) it can be suggested that global excretion of urolithin could eventually increase with periodically ingestion of BBJ.

The UPLC /ESI(+)-Q-TOF/MS profiling of tentatively assigned ETs metabolites in the urine collected during four day periods are reported in **Table 4.3**. The relatively lower presence of UC, UD, UM5, HHDP and EA derivatives could indicate that they are intermediate substrates with lower hydrophobicity than UA and UB, and therefore were poorly absorbed and excreted, remaining as a substrate for the microbiome in the intestinal lumen.

Despite ellagic acid (EA) being the first ET-derivative that appears during the digestion process in the stomach and duodenum [9], it was hardly detected in urine. Only as dimethyl ellagic acid (DMEA) or in its glucuronide form (DMEAG), it was found. This suggests that EA goes into the enterohepatic circulation where it is conjugated. EA can be methylated in the liver by catechol O-methyl transferase (COMT) and glucuronide [73] to facilitate urinary excretion. Urolithin C (aglycone and glucuronide forms) as well as other derivative forms (DMUC and UCMEDG) could be detected in all individuals. UD was also detected in all individuals (aglycone and glucuronide forms), even though it was at a lower level. Methyl urolithin A (MUA) was also detected in all individuals, but its base peak intensity was correlated mostly with UA derivatives (aglycone and glucuronide forms). Based on the UPLC /ESI(+)-Q-TOF/MS profiling, no other discriminating ETs metabolites could be evidenced between predominant UA and UB derivatives excreters.

Although the low presence of these metabolites and the lack of standards impede their quantification, it could be proposed that they are not able to reach the physiological concentration in plasma or target tissues to exert any protective health effects.

The urinary excretion profile appears to be consistent with previous studies [9, 14] that report the presence of EA, UM5 (3,4,8,9, 10-pentahydroxy-urolithin), urolithin D (tetrahydroxy-urolithin), urolithin C (trihydroxy-urolithin), urolithin A (dihydroxy-urolithin) and urolithin B (monohydroxy-urolithin) as metabolites resulting from the ETs degradation, nonetheless from this assay it was not possible to confirm the suggested

sequential dehydroxylation of EA by gut microbiota. As the inter individual variability had been already confirmed and also a discrimination between UA and UB excreters had been established, the next step consisted on finding out whether the inter-individual variability was also present in the kinetic of ETs degradation; and if so, look for the controlling step in the metabolic pathways. Consequently a more precise and frequent monitoring urine analysis was proposed, to approach to kinetic metabolism of ETs.

Table 4.3 UPLC /ESI(+)-Q-TOF/MS profiling of the main ETs metabolites excreted in urine during the 4 days after ingestion of blackberry juice for nine individuals (letters) and for different intervention periods (numbered in chronological order)

	UA+ UAG	UB+ UBG	UC+ UCG	UD+ UDG	UM5	HHDP	EA	DMEA	DMEAG	MUA	UCME	DMUC	UCMEDG	UDMEG
G1	+	-	-	-	nd	-	-	-	-	-	nd	-	-	-
G2	+++	-	+	+	nd	nd	-	++	++	+	nd	+++	+	++
G3	++	+	-	-	+++	+	+	++	+++	+++	++	+	+	nd
G4	+++	-	-	-	nd	nd	nd	-	nd	+	+++	-	+++	nd
R	+++	+	-	-	nd		nd	-	+	+++	+++	-	-	nd
B	+++	+	-	-	++	nd	+	-	+++	-	++	++	+	nd
D	+	-	-	-	nd	nd	nd	-	nd	+	-	-	-	nd
W	+++	-	-	-	-	nd	nd	++	nd	++	+++	+	+	nd
O	+++	+	+	-	-	-	nd	-	-	-	+	++	-	-
I1	-	+	+	-	-	nd	nd	-	-	-	-	-	-	-
I2	+	++	-	+		nd	nd	+++	++	+	-	+++	+++	+++
H1	-	+	-	-	-	nd	nd	-	-	-	nd	-	-	-
H2	+++	+++	+++	+++		nd	+++	++	++	++	nd	+	+	nd
H3	++	+++	-	-		nd	nd	+	nd	+++	+	-	+	nd
C1	-	+		-	+	-	nd	-	nd	-	nd	-	-	-
C2	+++	+++	+	+	nd	nd	+++	+++	++	+++	nd	+	+	++

+++ The highest excretion for each metabolite; ++ average excretion; +low excretion;- traces; nd Not detected.

4.3.2 KINETIC EXCRETION OF UROLITHIN A AND UROLITHIN B

To assess the kinetic excretion of ETs metabolism a more exhaustive urine analysis was performed since every urine excretion of each individual along the seven day after juice ingestion was analyzed independently. **Figure 4.7** depicts the kinetic excretion for the 5 individuals of these UA and UB derivatives in micromole equivalent UA and UB. The kinetic excretion in urine during the 7 days depicted clear inter-individual variability, with some individuals (H and F) reaching the highest peaks of urolithin excretion during the first 12 hours after juice ingestion, and others individuals (G, C) reaching maximum urinary excretion after at least two days. Additionally, it can be observed that the kinetic excretion of urolithin follows a wave-like trend, with high and low levels probably corresponding to

digestive cycles. In fact, glucuronide conjugates, such as UAG, have been found to be preferentially excreted in the bile of Iberian pigs [9].

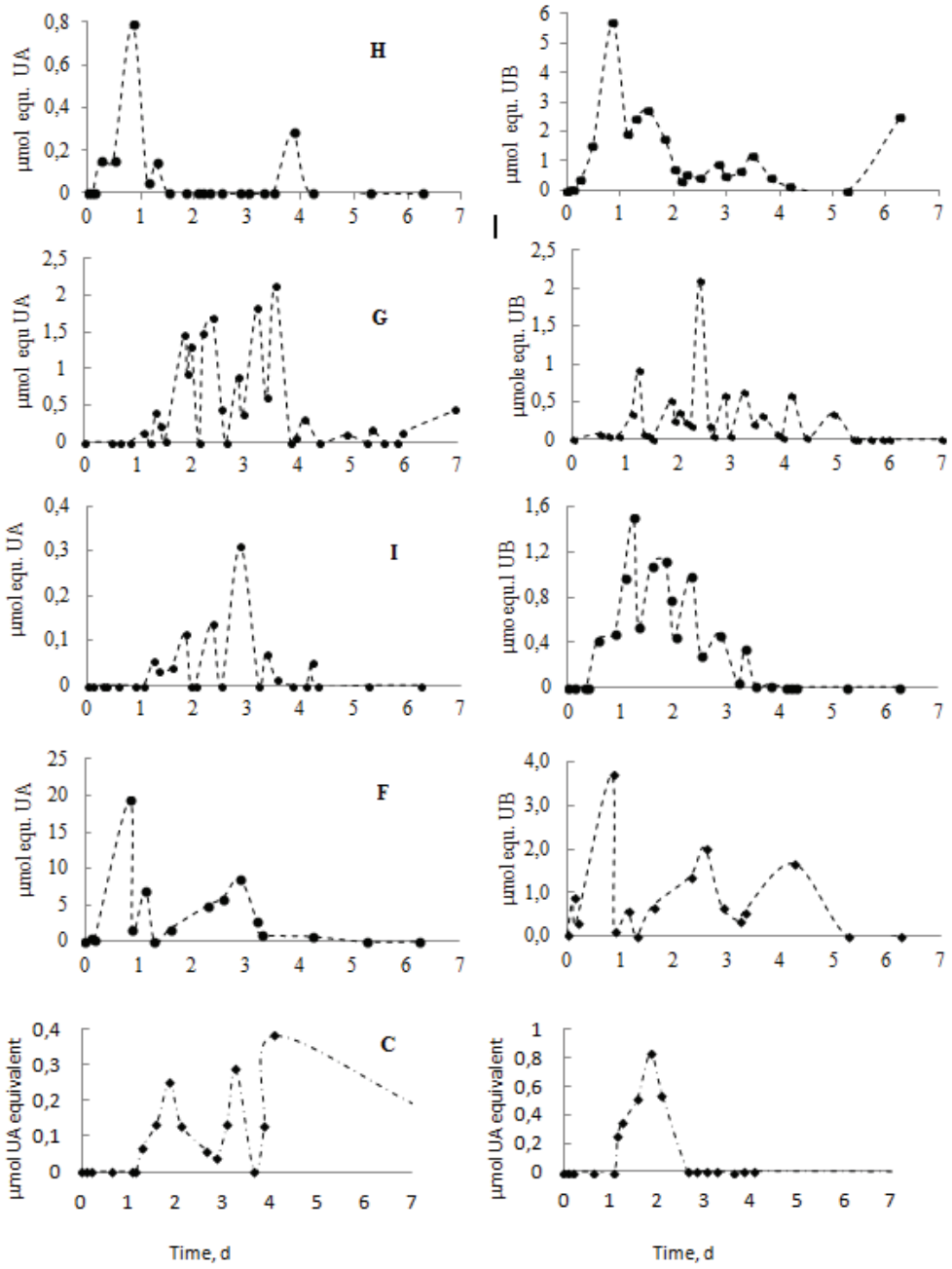


Figure 4.7 Urolithin UA and UB detected in every one of the samples of urine excreted for 5 individuals up to seven days after they ingested a 250-ml of BBJ.

The conjugates can then return to the small intestine and eventually undergo new bioconversion to aglycones before returning to the liver. This enterohepatic recirculation system, could explain the fluctuations observed and the relatively long residence time of UA and UB derivatives in the body.

The total excretion of UA and UB derivatives over the 7-day period is presented in **Table 4.4**. Total excretion of varied from 4 to 68 μmol equ UA/UB, after the ingestion of 250 ml of blackberry juice that contained 69 mg (228 μmol) of EA equivalents. Consequently, according to total urinary excretion, ETs' bioconversion into urolithins ranged from 1.9 to almost 30%. This relatively low global excretion in the urine can be explained by accumulation in some tissues, excretion in the feces and body fluids, degradation to other metabolites and degradation to lower mass compounds that were not identified in this study.

Table 4.4 Total excretion of urolithins UA and UB and percentage of conversion in relation to ETs ingested after seven days of BBJ ingestion.

Individuals	**Total amount excreted**			**% Eq. UA**	**% Eq. UB**	**% Conversion**
	UA derivatives (μmol Equ. UA)	**UB derivatives** (μmol Equ. UB)	**TOTAL UA/UB** (μmol Equ. UA/UB)			
G	15,15	8,35	23,50	64,47	35,53	10,29
H	1,74	26,93	28,68	6,09	93,91	12,55
C	1,81	2,52	4,34	41,77	58,23	1,90
I	0,83	9,55	10,376	7,98	92,02	4,54
F	55,03	13,05	68,08	80,83	19,17	29,80

The total excretion of UA and UB derivatives (**Table 4.4**) confirms that the individuals maintained their status as urolithins A (G, F) or as urolithins B (H and I) excreters. Individual C showed a low production of urolithins but maintain its status as UB excreter, since the total amount of UB derivatives excreted after the seven days was higher than those released of UA.

4.3.3 INTRA-INDIVIDUAL VARIABILITY OF URINARY EXCRETION OF UROLITHINS

To evaluate the intra individual variability of urolithins excretion, similar kinetics studies were carried out with four of these participants, analyzing all the urine excreted up to 4 days after volunteers ingested 250 mL of pure BBJ. This kinetic study was repeated several times along the year, with at least two month intervals of free living between interventions. **Figure 4.8** shows the total amount of urolithins released by day along the four days of the test.

First intervention trial (G1, H1, I1 and C1) correspond to results from the previous kinetic study in order to compare with the results with these new trials. Although the inter-individual variability in kinetic excretion was confirmed, the result that deserves mention is the high intra-individual variability found on the kinetics excretion of UA and UB derivatives, as the amount released of these metabolites by the same individual in the different assays varied significantly. Conversion rates tended to increase (except in the case of G4) after repeated intervention studies, although they were separated by at least two months of free living period for volunteers. This result could indicate a possible enhancement of an individual's response after repeated ingestion of ETs. Nonetheless, the status of "predominant UA or UB derivatives excreters" appears to remain constant, although UB predominance is less pronounced compared to previous results obtained on a single urine spot. This high intra individual variability could suggest that the categorization as high or low urolithins excreters should not be generalized, since the ability to release urolithins for a same individual is not constant, but it can change for different factors still unknown. Conversely to the clustering into UA or UB excreters that remain constant in the different trials carried out. In addition, this clustering could result interesting from the health point of view, since it seems that UA and UB exert potential benefits in different modes of action [14]

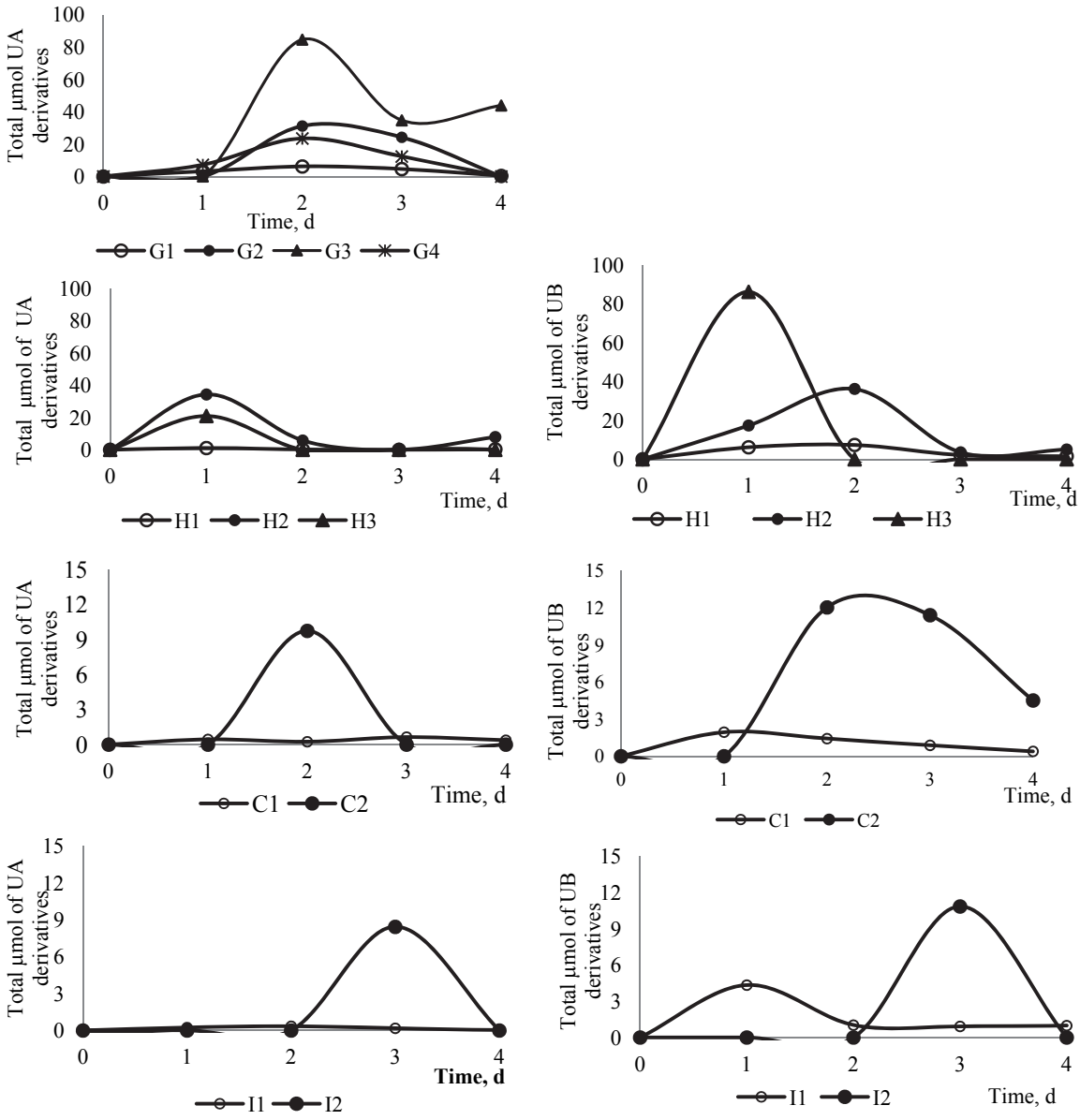


Figure 4.8 Excretion kinetics of UA(on the right) and UB(on the left) derivatives for four individuals (C,G,H, and I numbered in chronological order) performed at different periods of time around one year

4.4 IN-VITRO SIMULATION OF COLONIC FERMENTATION

In vitro models were carried out with the objective of corroborating and complementing the information obtained from *in vivo* trials regarding the ETs bioconversion patterns, the metabolic pathway of urolithins production and their relation with the kinetics of ETs degradation, the effect the substrate on the ability of microbiota to release urolithins and also the influence of BBJ on the ability to degrade ETs by microbiota intestinal.

First of all, batch culture incubations of BBJ inoculated with fecal stool from the same individuals that participated in clinical trials were carried out to evaluate if the results obtained from those models are maintained also *in vitro* trials, regarding the categorization in UA and UB excreters. Moreover incubations of EA and BBJ were also performed to find out the incidence of the substrate on the ability of microbiota to release urolithins and to approach to microbial route under which urolithins are released.

4.4.1 IDENTIFICATION OF ETs METABOLITES PRESENT IN FERMENTATION BROTH

UPLC-PDA/ ESI-Q-TOF/MS analysis of batch anaerobic incubation of blackberry juice (BBJ) and EA dissolved in DMSO lead to envisage eight metabolites according to their molecular weight (m/z^+) and UV spectra. The most important metabolites found in BBJ incubation was the EA (2,3,7,8- tetrahydroxy-chromeno[5,4,3-cde]chromene-5,10-dione) that was converted mostly into UA; whereas for EA incubations several metabolites were found, they were 3,4,8,9,10-pentahydroxy-urolithin (UM5), 2 isomers of urolithin D (tetrahydroxy-urolithin), 3 isomers of urolithin C (trihydroxy-urolithin) and urolithin A (3,8 dihydroxy-urolithin). Most of these metabolites can be observed in the UV chromatogram illustrated by **Figure 4.9.** The UPLC-PDA/ ESI-Q-TOF/MS analysis lead to identify these metabolites according to their chromatographic and MS spectra characteristics summarized in **Table 4.5**, and **4.6**. This figure depicts their UV spectra and their molecular structure.

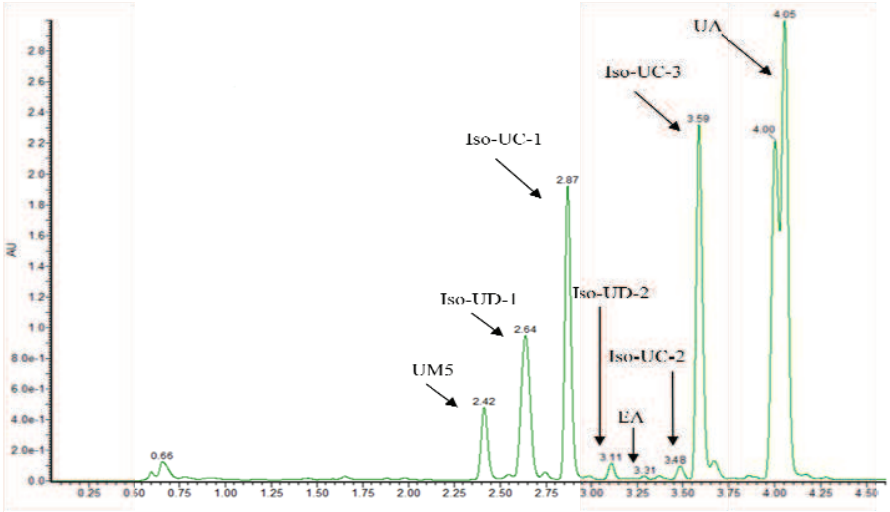
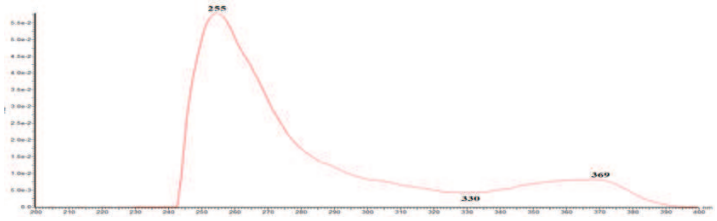
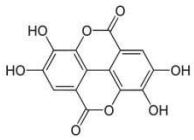
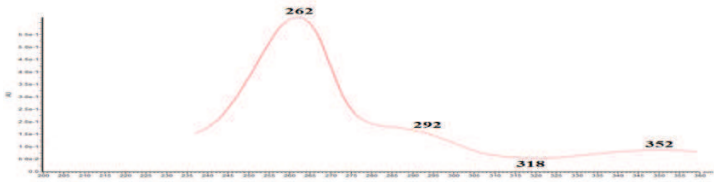
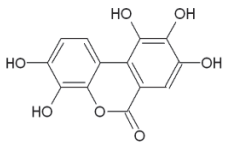


Figure 4.9 Example of UV chromatogram (at 278 nm) from a fermentation broth (EA as substrate, 120 h fermentation, stool 96 h inoculate from individual G

Table 4.5 Chromatographic and mass spectra characteristic of ellagitannins metabolites found in broth culture incubation of EA and BBJ inoculated with fecal stools from urolithin excreters

Rt (min)	UV spectra λmax (nm)	MS $(M+H)^+$	MS^2 m/z *(% base peak)*	Predicted log D	Assignment	Acronym
4.05	240,279,305,355	229.0501	185*(32)*,157*(100)*, 128*(55)*,*115(20)*	1.85	Urolithin A	UA
2.87	252,275,sh287,368	245.045	170*(100)*,142*(15)*,130*(30)*,115*(20)*	1.27	Iso-Urolithin C-1	UC1
3.48	267,sh295,355	245.045		1.59	Iso-Urolithin C-2	UC2
3.6	249,272,sh285,367	245.045		2.24	Iso-Urolithin C-3	UC3
2.69	253,sh303,340	261.0399	218(*65*),172*(85)* , 120*(85)*,114*(100)*	1.66	Iso-Urolithin D-1	UD1
3.11	259,sh288,347	261.0399		1.75	Iso-Urolithin D-2	UD2
2.4	262,sh292,352	277.0348	260(*50*),232(*48*)188(*100*),114(20)	1.48	Urolithin M5	UM5
3.31	255, 369	303.0141	131(100),177(55),153(45)	-0.72	Ellagic acid	EA

Table 4.6 Main ETs metabolites released along the degradation of ETs.a) EA, b) UM5, c) Iso UD1, d) Iso UD2, e) Iso UD3.

	
a)Ellagic acid. $C_{14}H6O_{8.}$ MW: 302.0062	
	
b)UM5: $C_{13}H_8O_7$.3,4,8,9,10-tetrahydroxy-6H-benzo[c]chromen-6-one)	

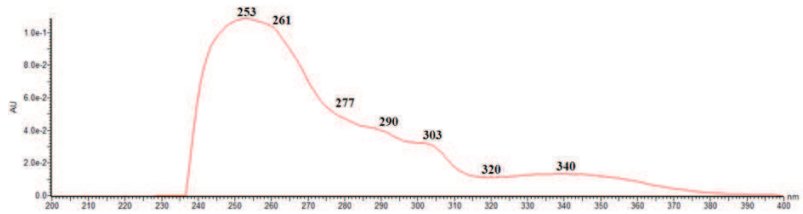
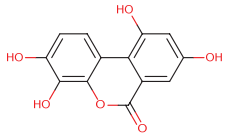
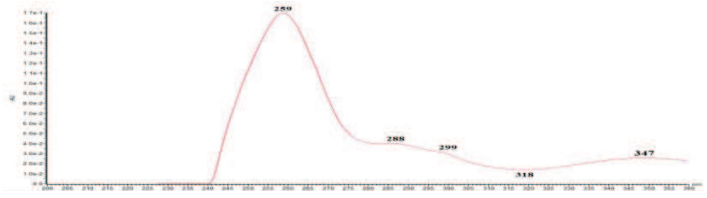
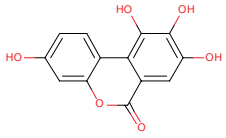
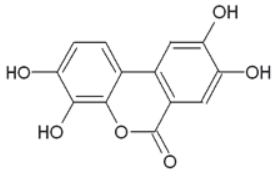
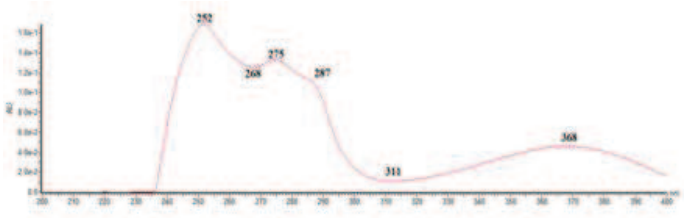
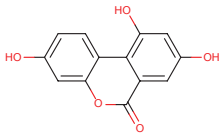
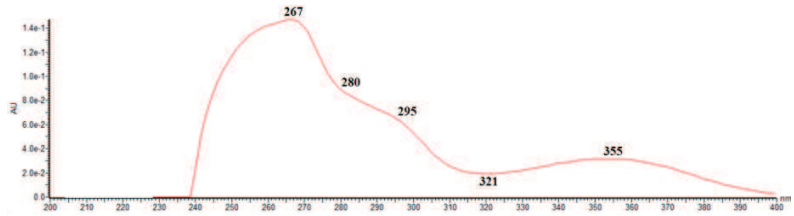
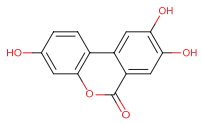
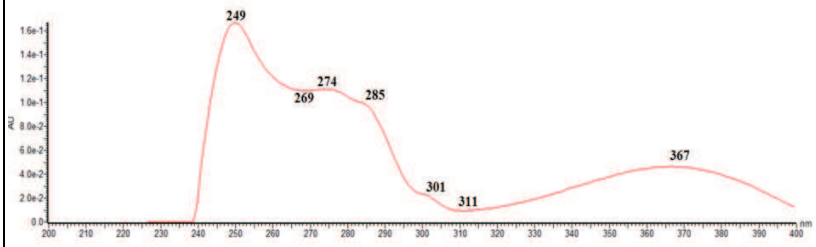
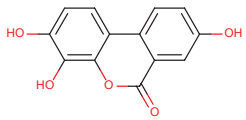
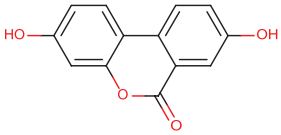
	
c) **ISO UD 1** :$C_{13}H_8O_6$ MW: 260.0321 Iso-urolithin D (**3,4,8,10**-tetrahydroxy-6H-benzo[c]chromen-6-one)	
	
d) **ISO-D 2**: $C_{13}H_8O_6$ MW: 260.0321. Iso-urolithin D (3,8,9,10-tetrahydroxy-6H-benzo[c]chromen-6-one)	
	NOT OBSERVED
e) **ISO UD 3.** $C_{13}H_8O_6$ MW: 260.0321: Iso-urolithin D (3,4,8,9-tetrahydroxy-6H-benzo[c]chromen-6-one	

Table 4.7 Main ETs metabolites released along the degradation of ETs. a)Iso UC1, b) Iso UC2, c) Iso UC3 d) UA

	
a) UC 1: $C_{13}H_8O_5$ MW: 244.061 Iso-urolithin C(3,8,10-hydroxy-6H-benzo[c]chromen-6-one)	
	
b)UC 2: $C_{13}H_8O_5$ MW: 244.06. Iso-urolithin C (3,8,9-hydroxy-6H-benzo[c]chromen-6-one	
	
c) **UC 3** : $C_{13}H_8O_5$ MW: 244.06 Iso-urolithin C (3,7,8-hydroxy-6H-benzo[c]chromen-6-one	
	
d)Urolithin A $C_{13}H_8O_4$ MW: 228.0423 (3,8-hydroxy-6H-benzo[c]chromen-6-one	

The characteristic UV spectrum of ETs metabolites and exact mass obtained by UPLC-ESI-Q-TOF/MS is in accordance with those previously published [58].

In vitro trials helped identify more ET metabolites than those found in clinical tests but in their aglycones forms; as is expected since conjugation reactions occur in liver or intestinal

cells. However one of the most interesting differences between these two models was the absence of UB aglycone from *in vitro* trials. It was surprising as UB had come out in clinical studies under its conjugated form of UB glucuronide and in such important amounts. These assays confirm the microbial origin of most of the urolithins.

The other point that can be highlighted is the differences in the number of metabolites released by incubation of EA and BBJ. For unknown reasons, EA can be degraded easily by fecal stool microbiota into diverse type of urolithins that finally lead to UA. However from BBJ the biodegradation route was not evident, since it was not possible to find any ETs metabolite different to EA in the broth culture until UA came out in the broth. Although there are no certainties in this finding, this could be explained in the fact that juice is rich in other compounds and intestinal microbiota has the ability to perform different types of reactions. Consequently different EA derivatives could be released and subsequently converted into other metabolites that could react with other compounds present in the juice that by further degradation release UA. However since no other EA derivatives were searched into the broth culture incubations it could be suggested that urolithins from BBJ also have microbial origins, but their metabolic pathways differ from those followed by EA and remain undefined.

4.5 VARIABILITY OF STOOLS' ABILITY TO PRODUCE ETS METABOLITES

The percentages of molar conversions of ETs or EA into urolithin metabolites, after 24h, 72 and 120 h of an anaerobic incubation of juice or EA with fecal stool of nine volunteers are depicted in **Figure 4.10**. At first glance, it can be observed the important variability between the ability of the different stools to produce ETs metabolites, which can also explain the high inter-individual variability observed *in-vivo* for the urinary excretion of urolithins derivatives.

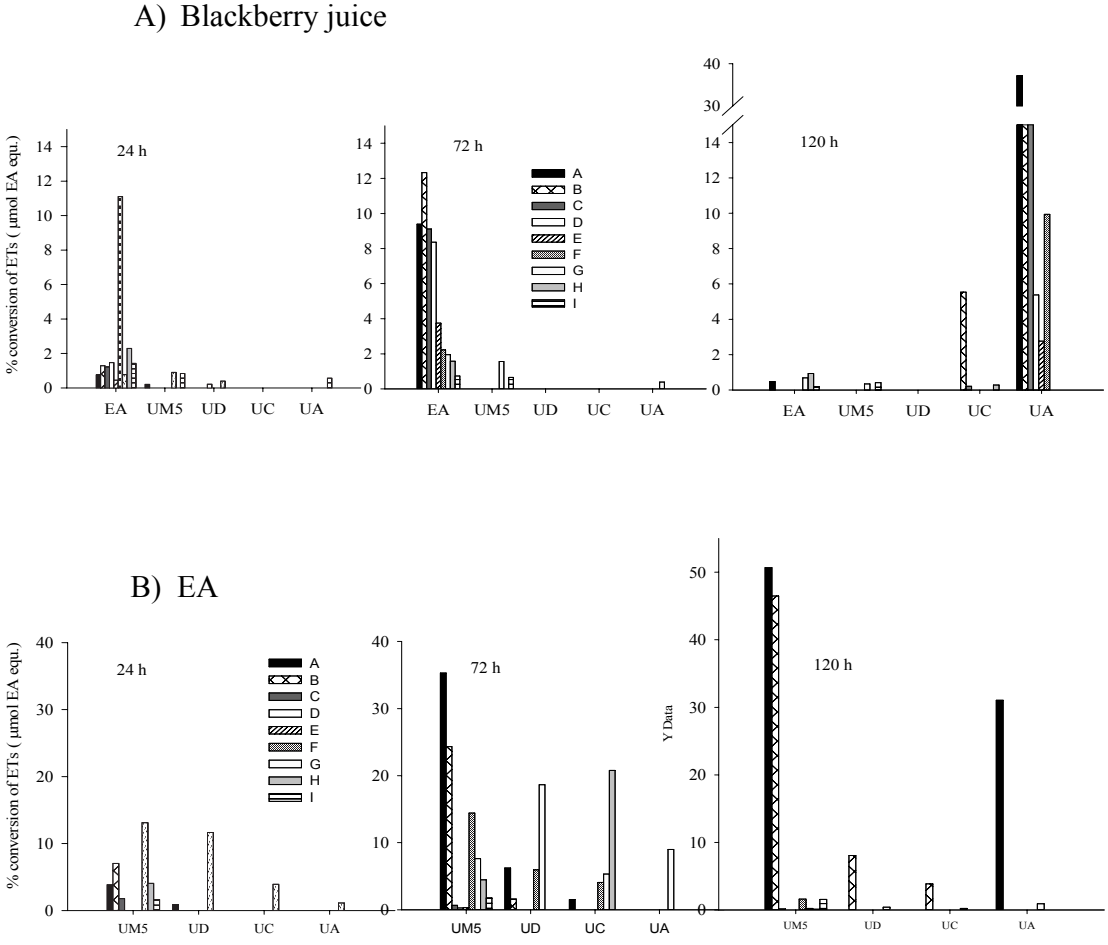


Figure 4.10 Primary ellagitannins metabolites assessed after 24, 72 and 120 h of batch anaerobic incubations of blackberry juice (A) and ellagic acid (B) with fecal stools donated by nine volunteers (A, B,C,D,E,F,G,H,I), previously qualified as urinary

4.5.1 EFFECT OF SUBSTRATE IN THE PRODUCTION OF UROLITHINS

From the same **Figure 4.10**, it is also possible to observe the effect of the substrate on the microbial ability to release urolithins. Comparing the two plots is evident the different pathway followed by fecal stool microbiota to convert EA and BBJ into urolithins and also the major activity showed by microbial community when EA is the substrate.

4.5.1.1 Urolithin production from BBJ

Figure 4.10A. shows that EA is easily released from BBJ, since at first 24 hours EA comes into the broth and at 72 hours reaches important amounts. Consequently, it can be stated that EA release is not a controlling step in BBJ conversion into urolithins. On the other hand all other metabolites hardly emerged in important quantities during the study. Only after 120 hours UA came out and became the most important microbial metabolite released by the ETs degradation. This means that EA is probably transformed into UA, following a metabolic pathway different to that proposed so far by some authors [9, 14, 121] or maybe the transformation of EA into other intermediate metabolites from these into UA is too fast to be followed in this trial.

Whatever the cause for the low presence of these metabolites, it is evident that they could hardly exert any systemic health benefits on the consumer, since in clinical studies they were not found in measurable quantities.

4.5.1.2 Urolithins production from EA

Figure 4.10B shows how the route to release urolithins depends on the substrate used. From this figure it can be said that degradation of EA is in accordance with the metabolic pathway proposed for some authors [9, 14] since the presence of UM5, UD, UC is evident until it reaches UA; whereas from BBJ only UA achieved high concentrations. This leads to think that bioconversion of EA into urolithins follows a different route to that pursued by ETs from BBJ. In addition, the same figure also shows UM5 is the predominant urolithin released from EA degradation even after 120 h of fermentation, which could lead to think that conversion of UM5 into other metabolites such as UD or UC could be the controlling step in the degradation of EA; whereas for ETs from BBJ, the conversion of EA into other metabolites could be the controlling step. According with this it could be suggested that EA is not released as free EA from BBJ, but as EA derivatives that are broken down in the cone of the MS releasing EA. Those EA derivatives can be also converted into urolithin A by colonic microbiota, and although their degradation takes longer time than free EA, the conversion into UA is also high.

Under conditions followed in the trials, it can be suggested that BBJ results a better substrate than EA for degradation by stool microflora, according to the UA conversion reached, despite the fact that no other ETs metabolites were found. The BBJ is a more suitable substrate for releasing UA than EA, as only the inoculums from one of the individuals had the ability to degrade the EA into UA, while the BBJ was degraded into UA by most of the stool. The fact that no other ETs metabolites were found from the BBJ degradation is not important as biological effects of ETs metabolites are due to UA and UB[14, 45, 54, 72, 75, 115, 117].

4.5.2 SUGGESTED BIOCONVERSION PATHWAY OF ETs TO UROLITHIN IN-VITRO

Fecal stools from 9 donors, previously selected for their ability to excrete urolithins in their urines, were used as inoculums during batch anaerobic fermentation of sterile blackberry juice and a solution of ellagic acid (EA). After 24, 48 and 120 hours of fermentation, broth cultures were analyzed by UPLC-DAD/ ESI-Q-TOF/MS for quantification of primary ellagitannins (ETs) metabolites. Eight metabolites were quantified by UV detector, ellagic acid (EA), 3,4,8,9,10-pentahydroxy-urolithin (UM5), 2 isomers of urolithin D (tetrahydroxy-urolithin), 3 isomers of urolithin C (trihydroxy-urolithin) and urolithin A (3,8 dihydroxy-urolithin). These results are in complete accordance with previous studies of ETs metabolism that pointed out these metabolites as the result of the ETs degradation. However, the metabolic pathway to transform ETs from BBJ into urolithins was not corroborated because none of these metabolites came out into the broth in quantifiable concentrations. From the results achieved by EA degradation by fecal stool a bioconversion pathway of EA into urolithins was proposed and presented in **Figure 4.11.** This metabolic pathway is in accordance with that suggested in previous articles [9, 14], although with some modifications concerning the inclusion of UC and UD isomers. Nevertheless, a gap concerning to metabolic route of ETs remains unsolved. Although it was evident that ETs is converted easily into EA, there are no certainties about the fate of this compound. A possible explanation proposed in clinical assays could be also valid in this *in vitro* model.

Probably also EA derivatives were released and their further degradation gave origin to UA. Unfortunately EA derivatives were not looking for in the UPLC-MS/MS analysis and then there are no proves for this hypothesis.

One the most remarkable results from the first *in vitro* assay is the absence of UB in all the batch cultures carried out, even more in those inoculated with FS from individuals previously clustered as UB excreters in *in vivo* assays. This fact leads to discuss the potential importance of the interactions between microbiota and host.

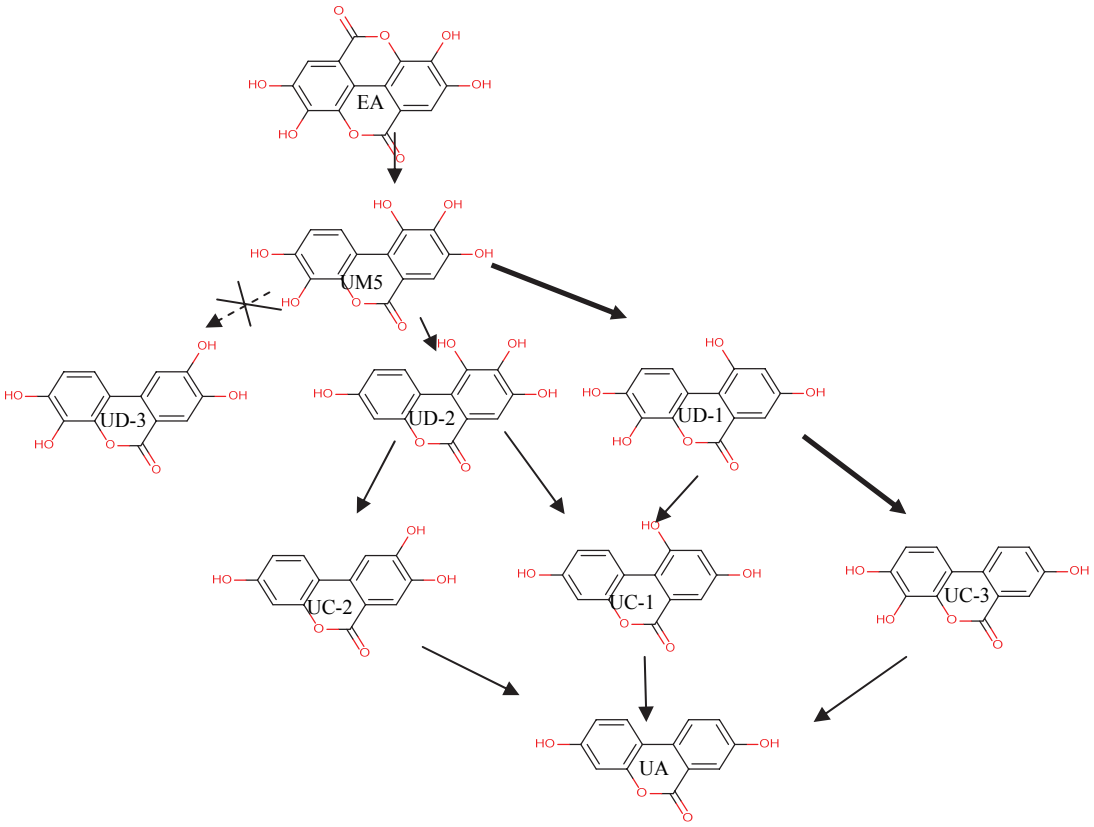


Figure 4.11 Suggested metabolite pathway occurring during incubation of pure ellagic acid with active fecal stool from urolithin excreter

4.6 POTENTIAL INTERACTION BETWEEN MICROBIOTA AND HOST

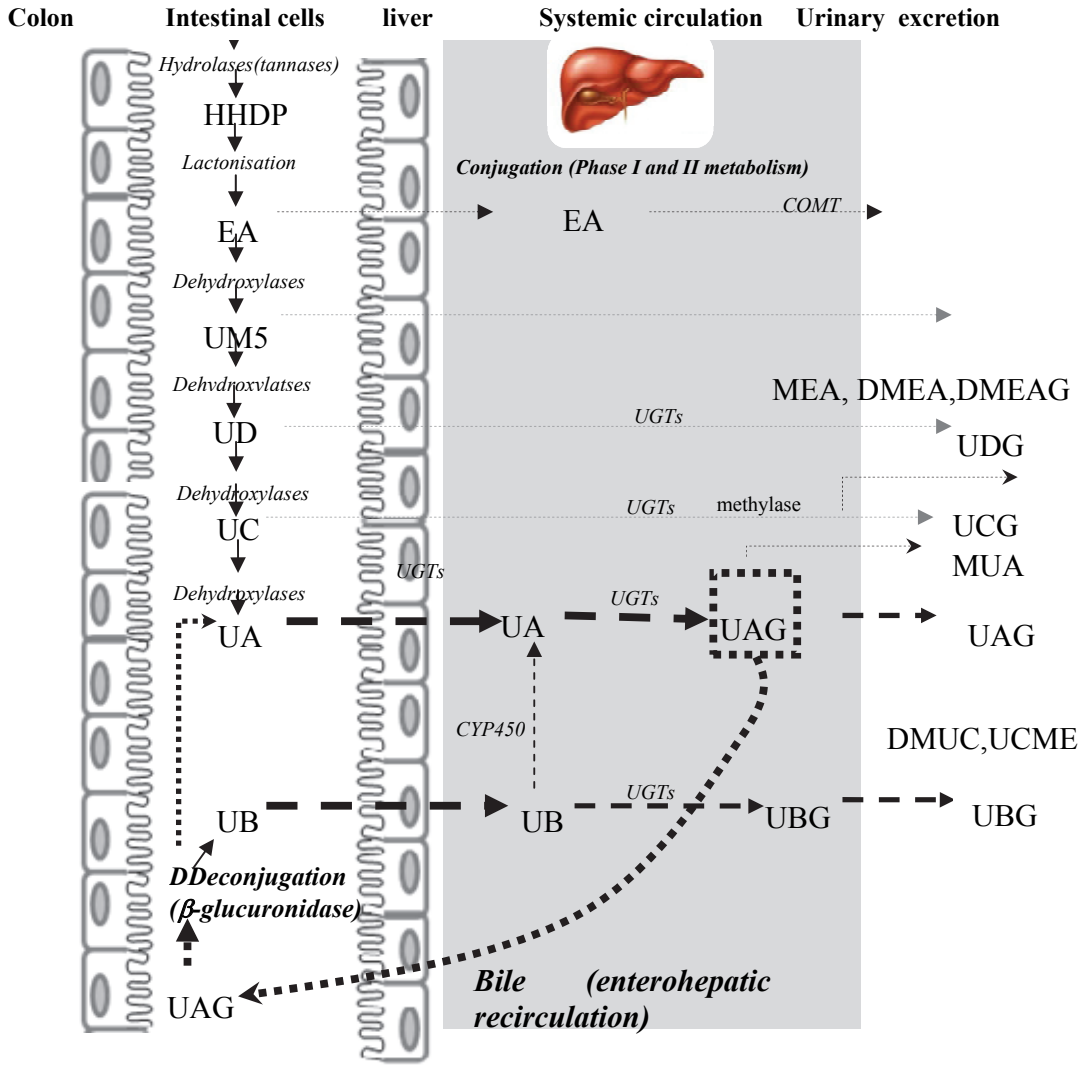
The urolithin UB that prevails during urinary excretion in some individuals was not produced *in-vitro* during batch culture with stools, even from UB derivative excreters. It could be possible that UB production require the participation of enzymes or physiological

conditions of the digestive system or maybe the microorganisms that participated in the UB production needs more appropriate conditions to grow than those used in the in vitro test. In addition, simulation of colonic fermentation *in-vitro* showed a slower bioconversion process of ETs and even EA into urolithin, than *in-vivo*. Actually, *in-vivo* urolithin production peak was reached between 40 and 60 hours after ingestion of juice. The gastrointestinal tract conditions, with constant mixtures and malaxations may facilitate the biodegradation of ETs by microbiota intestinal. However, we should have at least detected with fecal stool of UB excreters, (according to *in vivo* test) traces of urolithins UB. These stools were able to produce UA even more than *in vivo* which confirms unambiguously the microbial origin of UA, but not for UB. These results were observed for incubations with both substrate EA and ETs.

Comparing the results found in-vivo and *in vitro*, it can be proposed that ETs undergo both bioconversion by intestinal microbiota but also additional bioconversion by the metabolic system of host. The in-vivo trials where BBJ was ingested helped identify UB in important biological quantities, as well as other ETs metabolites in their conjugated forms, whereas *in vitro* trials from BBJ incubations results were different, since only UA came out in high concentrations. A hypothesis to explain these results should consider the possibility of an efficient recycling of UAG, which is excreted preferentially in the bile [9] and in the gut, where under microbiome action, simultaneous deconjugation and hydroxylation could be facilitated to yield UB in some individual.

This hypothesis is illustrated in **Figure 4.12**. In this case, UAG should be excreted first. However, according to our results, no evidence of lag time between the excretion of UAG and UBG was observed. Nonetheless, this time lag should be difficult to evidence in-vivo. Additionally, the human liver cytochrome CYP450 enzymes could be involved in the pathway as this enzyme has been previously shown to catalyze the hydroxylation of UB into UA to facilitate glucuronidation [73]. Actually, the excretion of UA derivatives should be facilitated over UB because of the presence of two hydroxyl groups that can be more easily glucuronided in the liver, compared to only one available hydroxyl group for UB. The same work [73] shows that the presence of UB enhances the expression of CYP450 genes 15- to 20-fold times in intestinal Caco-2 cells. As the polymorphism of cytochrome

CYP450 is relatively high between individuals [222], predominant excretion of UB derivatives could be due to lower activity of this enzyme.



COMT: catechol O-methyl transferase *CYP450: Cytochrome P450*

UGTs P-glucuronosyltransferases

Figure 4.12 Schematic representation of proposed metabolic pathways of the excretion of the main metabolites identified in human urine after ingestion of tropical highland blackberry juice

5 MICROBIOTA ECOLOGY AND ABILITY TO PRODUCE UROLITHINS

5.1 RESEARCH OF CORRELATION BETWEEN GUT ECOLOGY AND IN-VIVO ABILITY TO EXCRETE UROLITHINS

A major challenge to study human gut microbiota ecology is the assessment of the bacterial diversity or community structure present in the gastrointestinal environment. Most of the species present in gut microbiota cannot be cultured by classical method and culture independent techniques are required. Recent advances in molecular biology techniques are now becoming available and can be used to profile the microbial ecology of different environments and to try to evidence differences or changes in diversity, composition and structure. A case in point is the present study, which proposes to investigate if a relationship between gut microbiota ecology and the status of urolithin excreters of human volunteers studied previously can be evidenced. For this, we have chosen to use PCR-DGGE profiling, a molecular technique based on DNA analysis that has been widely applied to assess microbial ecology in different environments although it has been used rarely and only very recently for profiling gut microbiota [223]. The advantages of using DGGE are well established, as it provides a more accurate means of visualizing whole microbial communities. As it is based on DNA and multiplication by PCR, bias in the detection of unculturable species is reduced. Additionally, it is relatively easy to implement and much less labor-intensive than the more-conventional culture-independent techniques of PCR cloning and sequencing.

5.1.1 PCR-DGGE PROFILING OF THE 26 VOLUNTEERS

This first study aims at establishing the baseline for microbiota ecology fingerprinting of 26 volunteers who donated fecal stool before ingesting blackberry juice. The collection of stools was performed the same day of the ingestion of blackberry juice. The DGGE fingerprint of bacterial gut microbiota of the 26 volunteers is presented on **Figure 5.1** and

5.2, as an unweighted pair group method with arithmetic averages (UPGMA) dendogram. First, it can be observed a good repeatability between gels as DGEE profile of a same individual but obtained in different gels (Latin letter + number) clustered in the exact same sub-groups with very high similarities. In fact, one of the main drawbacks of gel electrophoresis techniques is the difficulty of comparing lanes between gels and taking all the precautions to perform the analysis in the same conditions, since important differences can appear. Nonetheless, thanks to the correction of background noise, alignment of the retardation factor (Rf), deconvolution of overlaid peaks and the calculation of band % which allows for correction of the loading and light exposure, it was possible to obtain reproducible results.

In **Figure 5.1**, we can observe that the banding patterns generated are quite complex, as 53 different bands could be detected. Even though this number is not representative of all the diversity of gut microbiota, DGGE fingerprinting should be considered representative of the most common bacteria in the human intestinal microbial community. Each gel lane corresponds to the DGGE separation of the amplicons of bacterial 16S rRNA genes and bands or "grey/black spots" corresponds to specific amplicons characteristics of a microbial species. Comparing by eye the 26 patterns in the bands proved difficult at first, and only a hierarchical cluster analysis allowed to distinguish 6 distinct groups (excepting control), at 60% dissimilarity threshold. We can also observe a large group that includes more than 50 % of individuals. Nonetheless, dissimilarity remains high among groups (**Figure 5.2**) and these results confirm the important variability in the composition and ecology of gut bacteria among humans.

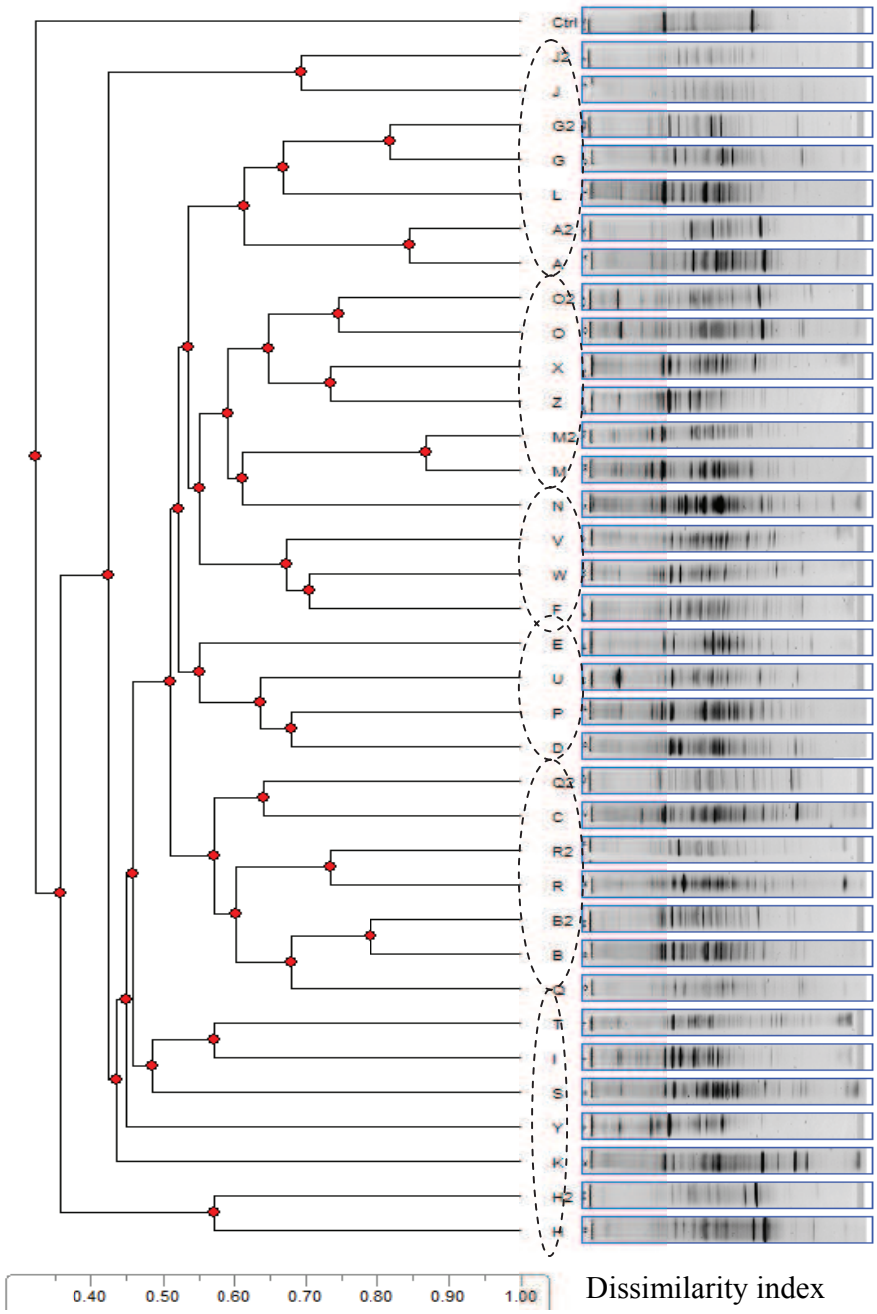


Figure 5.1 Hierarchical cluster analysis of the 26 PCR-DGGE profile PCR-DGGE fingerprint corresponding to amplicons of the V3 region of 16S rDNA of faecal bacteria from stool (Letter and number of lane corresponds respectively to volunteers identity and number of repetition; Ctrl is a control lane with *lactobacillus plantarum* and *E.Coli*)

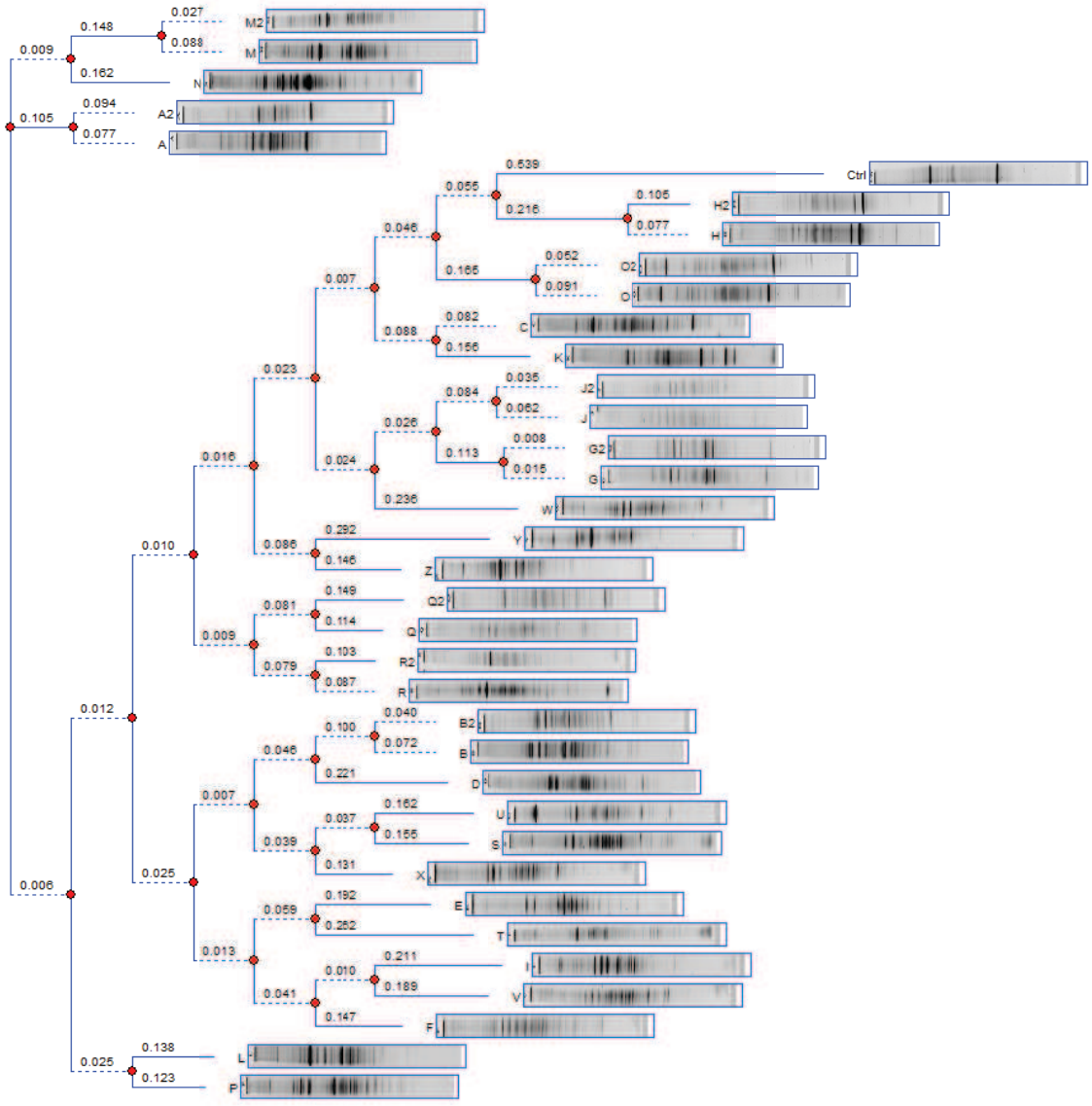


Figure 5.2 Complete linkage cluster analyses of 1-r (1 minus the Pearson coefficient between lanes) (Letter and number of lane corresponds respectively to volunteers identity and number of repetition; Ctrl is a control lane with *L. plantarum* and *E.Coli*)

5.1.2 RELATION WITH UROLITHIN EXCRETERS STATUS DETERMINED ON A URINE SPOT

In **Table 5.1**, is presented the concentration of primary urolithin A and B derivatives (aglycone and glucuronides forms) detected in a urine spot, from 26 volunteers, collected at 51±5 h after ingestion of 250 mL of blackberry juice diluted (50:50) with water. The objective of this study is to try to relate previous microbiota profiling to the ability to excrete urolithin in urine.

Table 5.1 Concentration of urolithin A and B derivatives in a urine spot collected at 51±5 h, after ingestion of one shot of 250 ml of blackberry juice.

Individual	Gender	Urolitin excreter status	µmol equ.UA.l^{-1}	µmol equ. UB.l^{-1}	TOTAL, µmol.l^{-1}
A	Female	UA	30.7	1.5	32.2
B	Female	UA	10.1	1.5	11.6
C	Female	UB	nd	77.2	77.2
D	Male	N	nd	nd	--
E	Male	UB	nd	23.9	23.9
F	Male	UA	58.6	1.5	60.1
G	Female	UA	215.9	99.7	315.6
H	Female	UB	nd	28.0	28.0
I	Male	UB	nd	27.7	27.7
J	Female	UA	6.7	1.5	8.2
K	Female	UB	nd	23.3	23.3
L	Female	N	nd	nd	--
M	Female	N	nd	nd	--
N	Female	UA	20.9	1.5	22.4
O	Female	UB	12.9	32.4	45.3
P	Female	UA	78.0	1.5	79.5
Q	Female	UA	3.5	1.5	5.0
R	Female	UA	8.9	nd	8.9
S	Female	UB	nd	17.0	17.0
T	Male	UB	nd	4.7	4.7
U	Male	N	nd	nd	--
V	Male	UB	nd	11.4	11.4
W	Male	UA	52.0	1.5	53.4
X	Male	UB	nd	14.0	14.0
Y	Male	UB	nd	7.4	7.4
Z	Male	UA	23.7	1.5	25.1

nd* means “not detected”

5.1.2.1 PCA analysis

First, a PCA analysis was conducted to observe the data and how they spread over the first two principal components (**Figure 5.3**) that represent more than 33% of the overall variability.

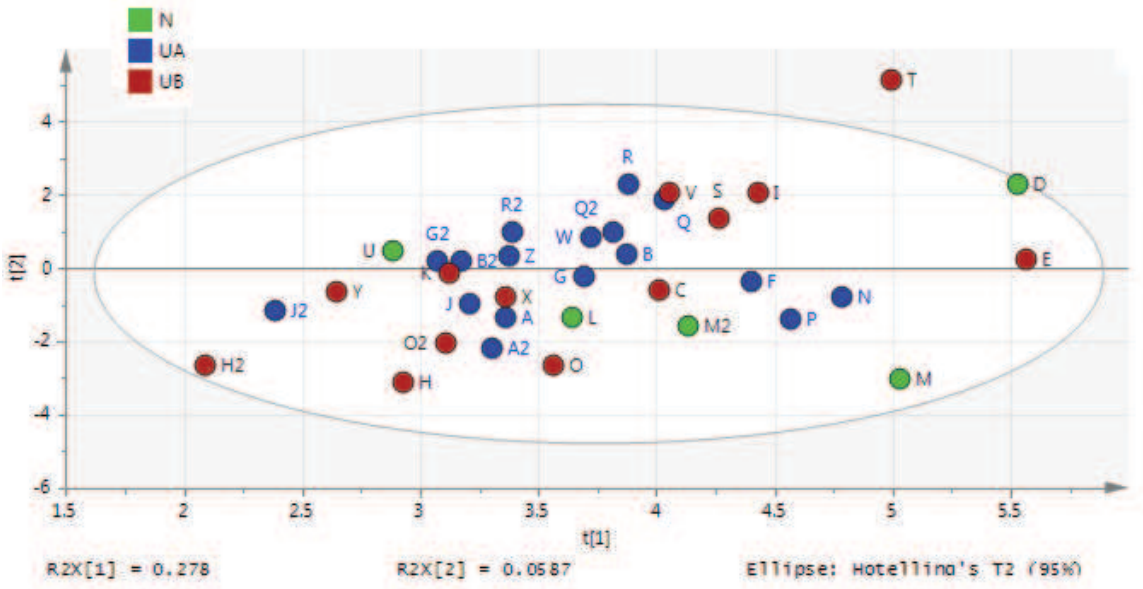


Figure 5.3 Principal Component Analysis (PCA) of PCR-DGGE profile for 26 volunteers and "urolithin excreters status" (N for "No urolithin excreters", UA for "Predominant UA excreters" and UB for "Predominant UB excreters".

It can be observed that PCA, at least on the first two principal components, does not allow observing clusters corresponding to the urolithin "excretion status" of the individuals. The relationship between DGEE profile and urolithin "excretion status" is probably linked to few bacteria which represent a relative lower variability compared to overall variability of the data. In this case, the Partial Least Square (PLS) regression is more appropriate to attempt to discover variability between given groups.

5.1.2.2 PLS-DA analysis

The PLS method allowed to find the multidimensional direction in the variable space X that explains the maximum multidimensional variance direction in the response space Y. In this approach, we decided to select as the response matrix, the urolithin excreters' status which is a discrete response that can have only 3 values "No urolithin excreters" (N), "predominant UA excreters" and finally "predominant UB excreters". Then, we defined 3 classes as response and a PLS-Discriminant analysis (PLS-DA) could be performed to assess correlation between a matrix variable [35 x 53] and a discrete response matrix [35 x 1]. The variable matrix [35 x 53], stands for 35 individuals (26 individuals + 9 repetitions) and 53 bands within the DGGE profile. Response matrix [35 x 1] stands for 35 individuals and 1 columns corresponding to 'urolithin excreters status". Results of PLS-DA analysis of the [35 x 53] matrix are displayed in **Figure 5.4** where the score scatter plot can be observed on the first two latent variables and **Figure 5.5** where it can be observed in the same space, the loading scatter plot of variables.

The PLS parametrical statistical tests (R^2Y_{cum}= 0.681 and Q^2_{cum}=0,5) and the ANOVA of the cross-validated residuals (CV-ANOVA) with a P-value = 0.003, were considered all acceptable for the validation of this PLS-DA model.

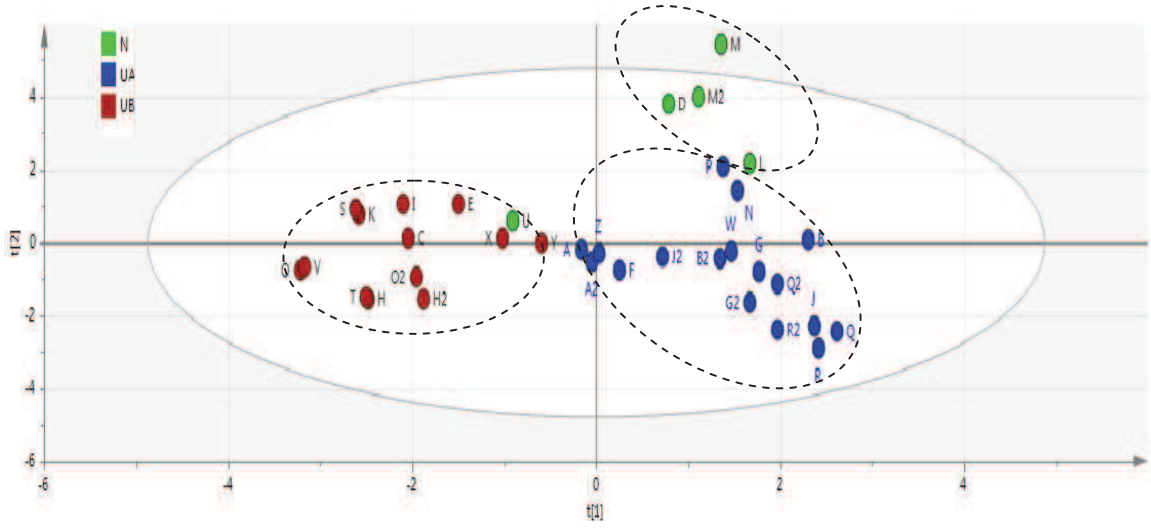


Figure 5.4 Score scatter plot of 26 individuals (plus some repetition) for PLS-DA regression of PCR-DGGE bands "X" taking as response ("Y") the urolithin excreters status (R2Xcum=0.146, R2Ycum=0.68, Q2cum=0.49, CV-ANOVA<0.05).

It can be observed that the PLS-DA analysis allows discrimination, except for individual U, between urolithin excreters status only basing discrimination on 14,6 % of the overall variability of variables (R^2X_{cum}=0.146). In the case, of individual U, it should be useful to reconsider his urolithin excretory status, as this determination on a single urine spot could be misleading but unfortunately, reassessment was not possible.

The loading plot of the band of the PCR-DGGE lanes presented in **Figure 5.5** shows the importance of some bands in the discrimination of the 3 different groups, "No urolithin excretors" (N), "predominant UA excretors" and finally "predominant UB excretors".

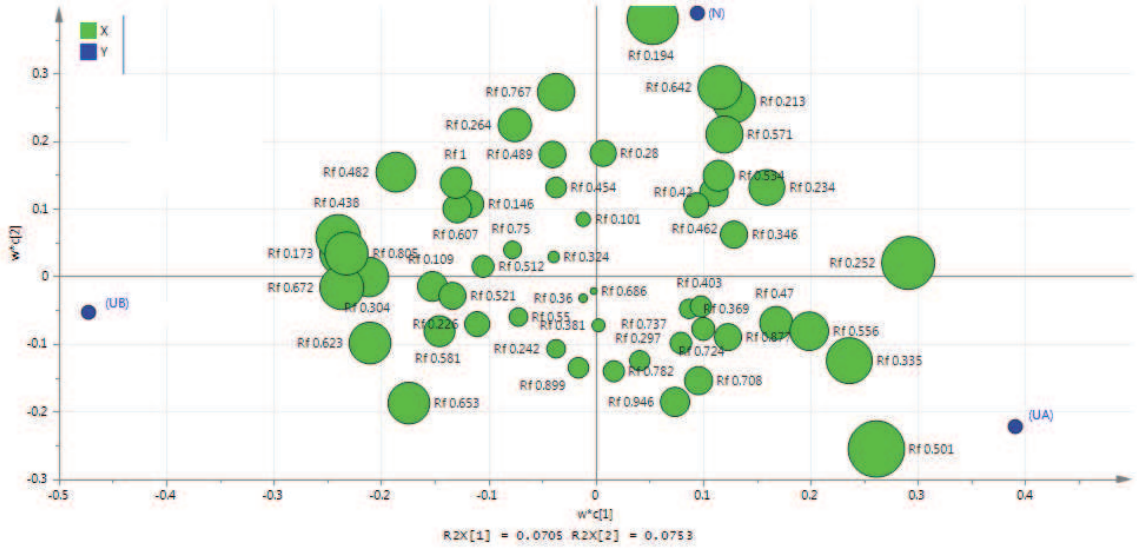


Figure 5.5: Loading plot of the different bands from PCR-DGGE analysis "X" for PLS-DA regression of PCR-DGGE bands "X" taking as response ("Y") the urolithin excreters status (R2Xcum=0.146, R2Ycum=0.68, Q2cum=0.49, CV-ANOVA p-Value =0.003) (Size of "X" variable

For the "No urolithin excreters" (N) group it appears that bands at Rf 0.194, 0.642 and 0.213 are the most important terms in the model with respect to the discrimination of this group. Nonetheless, the PLS-DA regression coefficients for each band displayed in **Figure 5.6** showed a too high confidence interval including zero value. For the group corresponding to "predominant UA excretors", results appear to be more reliable with lower confidence intervals of the regression coefficients of the most important terms in the model. For this group, we can observe 5 bands that appear to be related with UA excretion

patterns (Rf 0.501; 0.335; 0.252, 0.556 and 0.47). In the case of the third group corresponding to “predominant UB excretors”, there are also 5 bands correlated to this group (Rf 0.672; 0.438; 0.173; 0.805 and 0.623). All these bands should correspond to the sequence of some microorganisms that we will try to tentatively identify later.

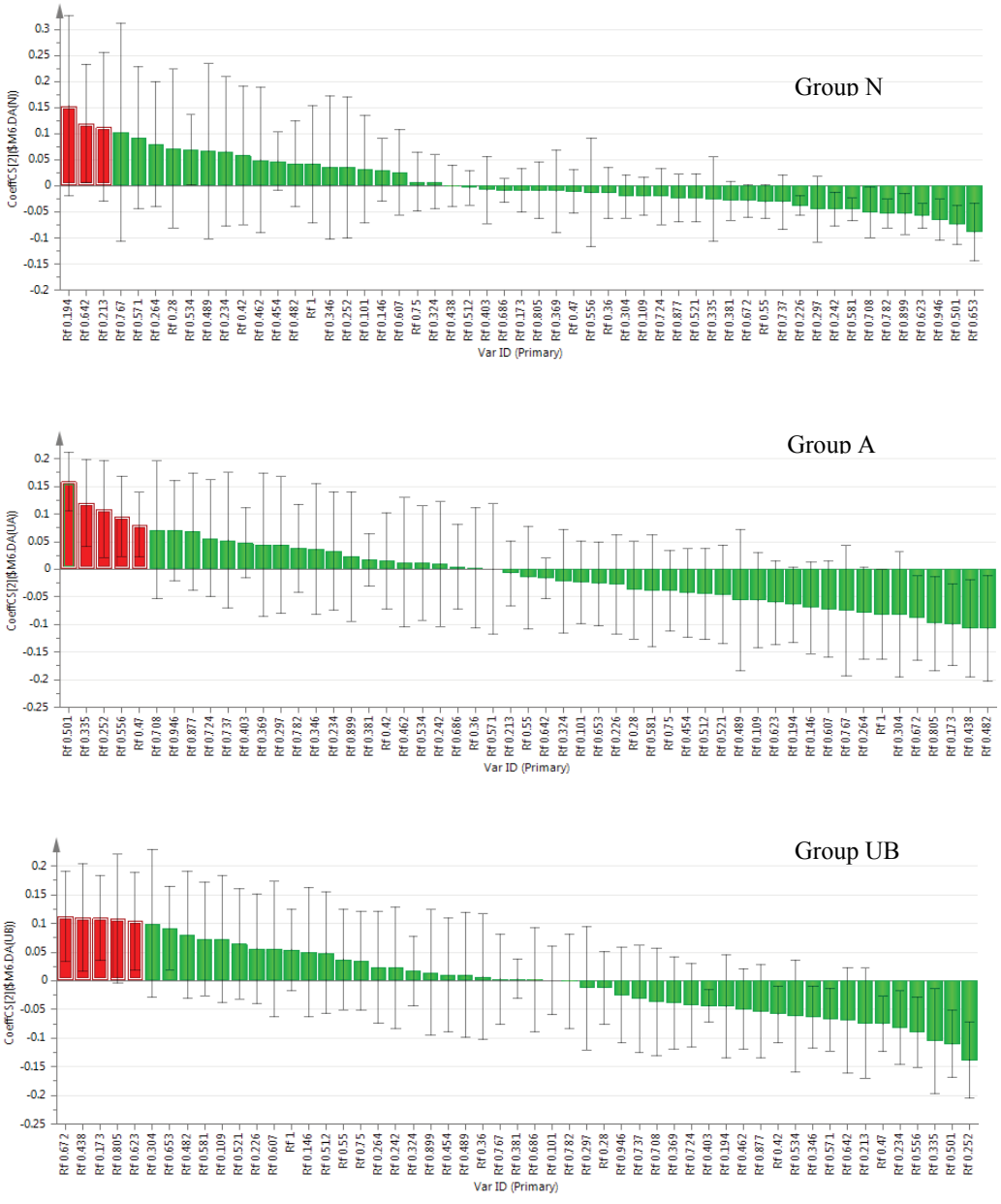


Figure 5.6 PLS-DA regression coefficients with confidence intervals for the three different groups, for each bands of the PCR-DGGE

Also, it should be mentioned that some bands were also negatively correlated with groups such as Rf 0.482 for UA group.

5.1.2.3 PLS analysis

This first part of this study was performed taking into account only the urolithin excreters' status, and PLS-DA method was used to discriminate the three groups. Nonetheless, the relative intensity of urolithin excretion could also be taken into account. Some individuals excrete more or less urolithin in their urine after blackberry juice consumption (**Table 5.1**) and this intensity may also be related to a specific composition of gut microbiota. For this task, a PLS analysis was performed to try and relate two data matrixes, the same [35 x 53] variable matrix as before and a response matrix [35 x 3], 3 standing for the concentration value of UA, UB and total urolithin in the spot of urine of the 26 individuals +9 repetitions.

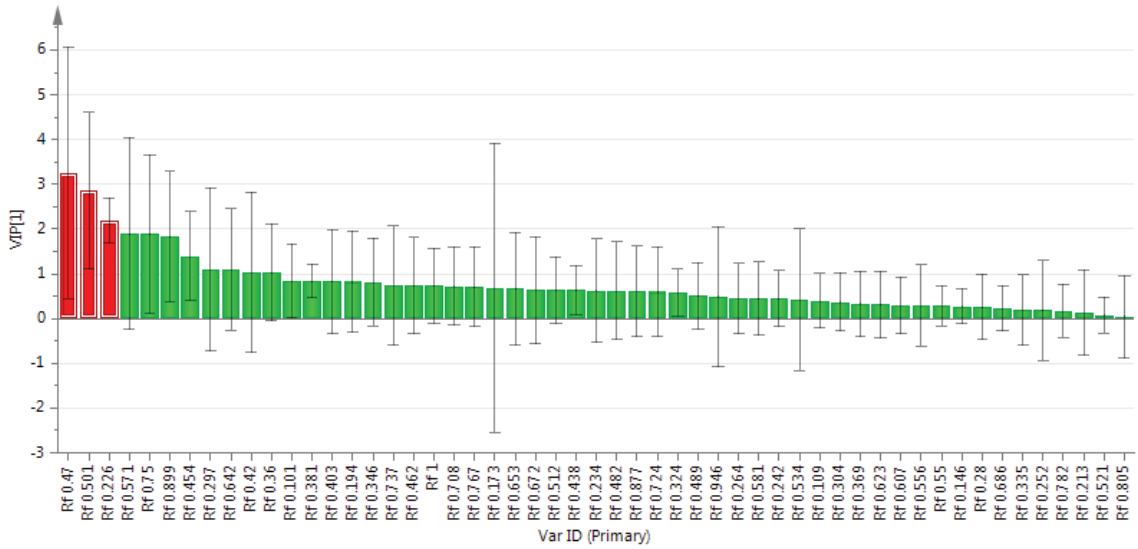


Figure 5.7 PLS variable importance for projection for each bands of the PCR-DGGE with confidence intervals (R2Xcum=0.0525, R2Ycum=0.6, Q2cum=0.39, CV-ANOVA for UA P-value =0.12, for UB 0.038 and for total urolithin 0.048)

The model was validated for UB, and total urolithin, but not for UA for which the P-Value of the ANOVA of cross-validated residuals was higher than the 0.05 threshold chosen for statistical significance. As it can be observed in this PLS model, **Figure 5.8**., the most

important bands were Rf 0.47; Rf 0.501 and Rf 0.226. The first two were already listed among the most important related with "predominant UA excreters" and Rf 0.226 was not considered previously, but according to this new PLS model, this band is correlated with increasing concentration of urolithins B in the urine spot.

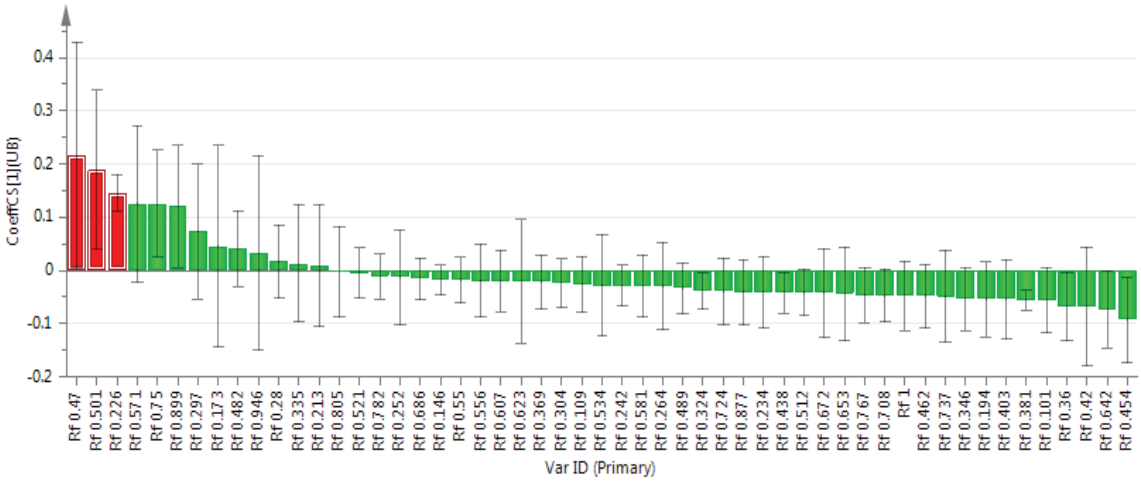


Figure 5.8 PLS regression coefficients for the "UB predominant excreters" group.

5.2 RELATION BETWEEN MICROBIOTA AND *IN-VITRO* BIOCONVERSION OF UROLITHIN

The stools donated for DGGE profiling were also preserved and used for the simulation of colonic fermentation. The study on the *in-vitro* simulation of colonic fermentation (&4.5) allowed us to determine an index describing the ability of microflora to metabolize ETs into EA (EA index) and into urolithin (UR index) when blackberry juice is used as substrate. EA and UR index are presented in Table 5.2 (calculation presented in & 3.2.4.7). Briefly, EA index is the percentage of bioconversion of ETs to EA after 72 h of anaerobic fermentation of blackberry juice for a given stool inoculum. Urolithin index is the sum of percentage of bioconversion of ETs into UM5, UD, UC and UA after 120 h of anaerobic fermentation of blackberry juice with a given stool inoculum.

Table 5.2 EA and UR Index of stools from 9 individuals

Individual	Genre	Sp. Status*	EA index	UR Index
A	Female	UA	9,39	37,15
B	Female	UA	12,33	34,17
C	Female	UB	9,12	28,95
D	Male	N	8,36	5,38
E	Male	UB	3,75	2,77
F	Male	UA	2,23	9,94
G	Female	UA	1,95	0,35
H	Female	UB	1,58	0,28
I	Male	UB	0,74	0,42

*Previous status *in vivo* on one urine spot (UA "predominant UA excreters", UB "Predominant UB excreters", N "no urolithin excreters")

An orthogonal partial least square (OPLS) regression was performed to assess correlation between the variable matrix [13 x 53] and the response [13 x 2] matrix, comprising the two bioconversion ability indexes, EA index and urolithin index. Results of OPLS analysis of the [9 x 82] matrix are displayed in **Figure 5.8**. The OPLS parametrical statistical tests were considered acceptable to use this model, even though for EA the P-value for the ANOVA of the cross-validated residuals model was slightly higher than the 0.05 threshold. The scatter plot of individuals and the loading plot of the PCR-DGGE bands referenced by their retardation factor on the lanes (Rf) are presented, as well as their regression coefficient within the model, including respective confidence intervals.

On **Figure 5.9**, at least 4 bands were identified with the highest variable importance for projection of the model (VIP) superior to 1 and with relatively reduced confidence intervals. Two bands (Rf 0.346; 0,252) were more positively correlated with urolithin indexes, while the same bands plus two other bands (Rf 0,724 and 0.556) were more correlated to EA index. It is also worthy to mention that Rf 0.899 and Rf 0.304 were negatively correlated with both index.

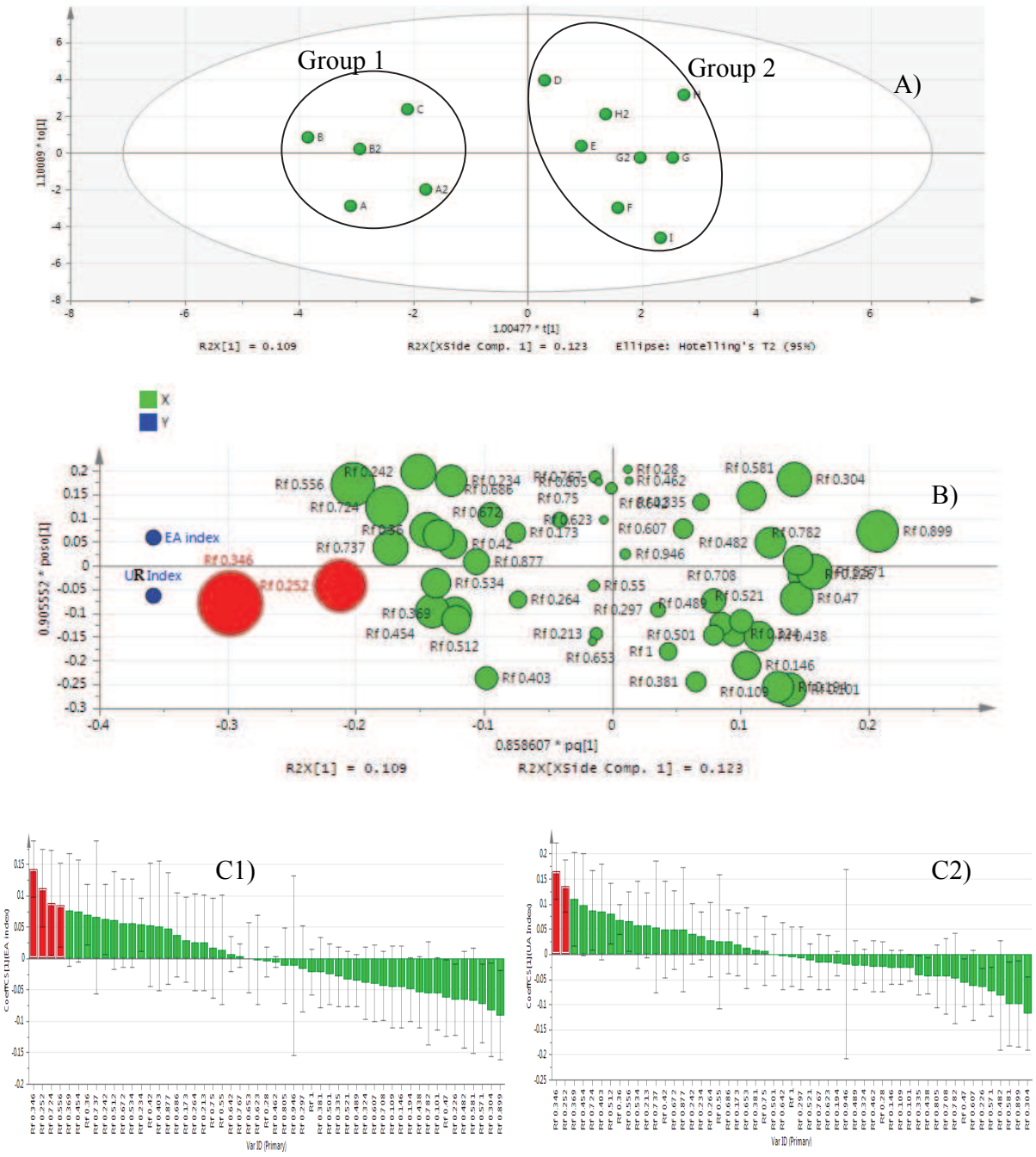


Figure 5.9 Scatter plot (A) and Loading plot (B) of PCR-DGGE bands "X" (I) and regression coefficients with confidence interval (C) obtained by OPLS taking as response ("Y"), bioconversion ability index of ellagitannins into ellagic acid (EA index) (C1) and into urolithin (UR index) (C2) (R^2X_{cum}=0.232, R^2Y_{cum}=0.93, Q^2_{cum}=0.60, CV-ANOVA p-value for EA index and UR index P-value 0.07 and 0.04 respectively) (In graph I, size of loading plot is proportional to "X" variable importance for projection of the model)

5.3 IDENTIFICATION OF FECAL BACTERIA INVOLVED IN UROLITHIN METABOLISM

The results of the different statistical analyses performed are summarized in **Table 5.3**, taking into account the urinary excretion status of individuals and the ability of their stools to produce urolithin. These analyses have highlighted different bands that are correlated to the observed responses.

Table 5.3 Recapitulation of retardation factors (Rf) of the bands found as discriminative in the different trials

PLS-DA (urine spot/ 26 individuals)						PLS (urine spot/ 26 individuals)		OPLS (Fecal stools)			
Group N		Group UA		Group UB		UB		Ea index		UA index	
+	-	+	-	+	-	+	-	+	-	+	-
Rf 0.194 0.642 0.213	Rf 0.653 0.501 0.946	Rf 0.501 0.335 0.252 0.556	Rf 0.482 0.438 0.173	Rf 0.173 0.438 0.672	Rf 0.252 0.501 0.335	Rf 0.47 0.501 0.226	Rf 0.454 0.642	Rf 0.346 0.252 0.724	Rf 0.899 0.304 0.571	Rf 0.346 0.252 0.369	Rf 0.304 0.899 0.581

A band (Rf 0.252) appears to be relevant both in *in-vivo* and *in vitro* trials. Another band Rf 0.501 is repeated in more than one test. Nonetheless, all the relevant bands were excised and sequenced to annotate their identity after querying the BLAST database for sequence similarities matching. Some bands could not be identified because of the lack of purity of the excised material. Nonetheless, most of them were identified. Results of the database query are presented in **Table 5.4** with respective scores.

Table 5.4 Results of the sequence similarities of the excised bacterial bands from different DGGE gels

Accession	Annotation	Rf	Max Score	Total score	Query coverage	E-value	Identity
NR_043317.1	*Parabacteroides goldsteiinii*	0,173	313	313	100%	9,00E-86	99%
DMS 17855	*Bacteroides dorei*	0,252	316	316	100%	7e-87	100%
ATCC 29863 DSM 6740	*Flavonifractor plautii / Clostridium orbiscindes*	0,335	279	279	95%	9e-76	95%
ATCC29799	*Bacteroides capillosus*	0,335	279	279	95%	9e-76	95%
NR_025421	*Limnobacter thiooxidans CS-K2*	0,369	209	209	97%	9e-55	94%
ATCCA BAA835	*Arkkemansia muciniphila*	0,454	322	322	91%	2e-88	99%
DSM9787	*Pseudobutyrivibrio ruminis*	0,470	220	220	100%	3,00E-58	100%
ATCC 27768	*Faecalibacterium prausnitzii*	0,501	313	313	100%	8e-86	100%
YIT 11860	*Barnesiella intestinihominis*	0,556	307	307	99%	5e-84	95%

The Bands Rf 0.252 and Rf 0.501 which appeared as the most relevant ones correspond respectively to *Bacteroides dorei* and *Faecalibacterium prausnitzii* with 100 % similarity of the amplicons of the V3 region of 16S rDNA. Both bacterial species seem highly correlated with *in-vivo* urinary excretion of urolithin and *in-vitro* degradation of ellagitannins from blackberry into ellagic acid (EA) and urolithins.

The genus *Bacteroides dorei* are commensal bacteria quite abundant in human microbiota, and they seem to be linked to healthy bowels as they benefit the host by excluding potential pathogens from colonizing the gut. They are bile-resistant [224] and consequently they could possibly use substrate of host origin or dietary origin but absorbed by host and redirected to gut by enteropathic recirculation system, as in the case of urolithin A glucuronide (UAG) which could be converted by this microorganism in UB.

Faecalibacterium prausnitzii (Rf 0,501) is one of the most abundant commensal bacteria in the healthy human large intestine as its concentration is reduced in certain forms of inflammatory bowel disease [225]. Additionally, it has been shown recently to be involved in the degradation of pectin and uronic acids, an ability previously thought to be confined to *Bacteroides spp* among human colonic anaerobes [226]. The function of *F. prausnitzii* in intestinal health could explain its relation to the higher ability for metabolizing ETs. It does

not mean necessarily that this micro-organism is directly involved in the metabolism, but it remains a potential candidate as it appears to be relatively versatile in the use of dietary and host origin substrate [226].

Other species highlighted are the *Flavonifractor plautii/clostridium orbiscindes* and *Bacteroides capillosus* (Rf 0,335) all three identified with the same 95% similarity of amplicons. *Flavonifractor plautii* synonym *clostridium orbiscindes* has been shown to degrade most flavonoids [227] by cleaving the flavonoid C-ring. Nonetheless, it could not utilize genestein or daidzein as substrates. *Bacteroides Capillosus* is a member of the division Firmicutes but no information on their substrate is available. Unfortunately very little is known on gut bacteria and this is the same for the bacteria *Parabacteroides goldsteiin* (Rf 0.173), *Barnesiella intestinihominis* (Rf 0,556) *Pseudobutyrivibrio ruminis* (Rf 0,470) and *Limnobacter thiooxidans* (Rf 0,369) that apparently play a role in ellagitannins bioconversion to urolithin as highlighted by our analysis.

On the other hand, *Akkremansia muciniphila* (Rf 0,454) was correlated with lower production of UB derivatives. *Akkremansia M.,* is a strictly anaerobic mucin-degrading bacterium. Resides in the mucus layer and comprises about 3% to 5% of the ileum and colon microflora. *A. muciniphila* has showed normalize appetite and metabolism, and also improve insulin resistance and fasting blood-glucose [228, 229]

5.4 EFFECTS OF ETS INGESTION ON MICROFLORA PROFILE

To explore the effect of blackberry ETs on the activity of intestinal microbiota to degraded ETs, a short test was performed. Volunteer G, who belongs to group 2 characterized by a low EA and Urolithin index was chosen to evaluate whether consumption of blackberry juice could enhance bioconversion ability and observe if this could be reflected on the PCR-DGGE fingerprint of stools. Fecal stools donated by individual G at 48h and 96h after ingesting a single shot 250-ml of BBJ were used as starter in the incubation of EA or BBJ in anaerobic conditions. Urolithin index (% conversion of EA or ETs into urolithins) was assessed after 120 h of incubation. Results of percentage bioconversion are shown on **Figure 5.10**, where it can be observed that stools collected at 96 hours after BBJ

consumption is much more active (urolithin index=8,4%) than the stools collected after 48 hours (urolithin index =1,4%), and much more than previous experiments (urolithin index 0,5%), in which the stools were collected after 7 days of washout period and just before juice ingestion. Bioconversion of EA to urolithins was also considerably enhanced reaching up to 50% using stools collected after 96 hours after juice ingestion. It is important to highlight that in controls cultures inoculated with these stools it was not found urolithins

Figure 5.10 also reports the PCR-DGGE fingerprints of the different stools. Visual examination of bands previously identified as discriminative, lead to the observation of increasing intensity and size in some bands, specifically for band Rf 0.252, Rf 0.470 and Rf 501 indicating a relatively higher concentration of amplicons corresponding to these microorganisms. They are *Bacteroides dorei (Rf 0.252)*, *Faecalibacterium prausnitzii (Rf 0.501)* and *Pseudobutyrivibrio ruminis (Rf 0,470).*

The PCR-DGGE fingerprints of stools, collected at different times after juice ingestion of three more individuals (individual F, I and C) are presented on **Figure 5.11**. From this figure, it is possible to observe changes in the intestinal microbial profile and especially and according to individual, for band Rf 0.173 (individual F and I for 72 h stool), for band Rf 0.252 (Individual F and C), band Rf 0,335 (individual I), band Rf 0,470 (Individual I and C), band Rf 0,501 (Individual C) and finally band Rf 0,556 (individual F and C). The activation of some of the bands highlighted previously is remarkable and confirms previous results despite the large variability between individual, which could be also caused by the small differences in the diet followed by the participants. For example proportion of *Bacteroides dorei (Rf 0.252)* seems to be enhanced for individual F and C, but not for individual I. Individual I belongs to the "No urolithin excreters" group. Probably because of the low proportion of this bacteria in the gut of individual C., the ingestion of blackberry juice not …… be activated sufficiently after On the other hand, it appears that *Flavonifractor plautii/clostridium orbiscindes* or *Bacteroides capillosus* (Rf 0,335) increases considerably instead, proving that microbiota adapt to substrate in different ways. Similar conclusions can be drawn with *Pseudobutyrivibrio ruminis* (Rf 0,470) and *Barnesiella intestinihominis* (Rf 0,556).

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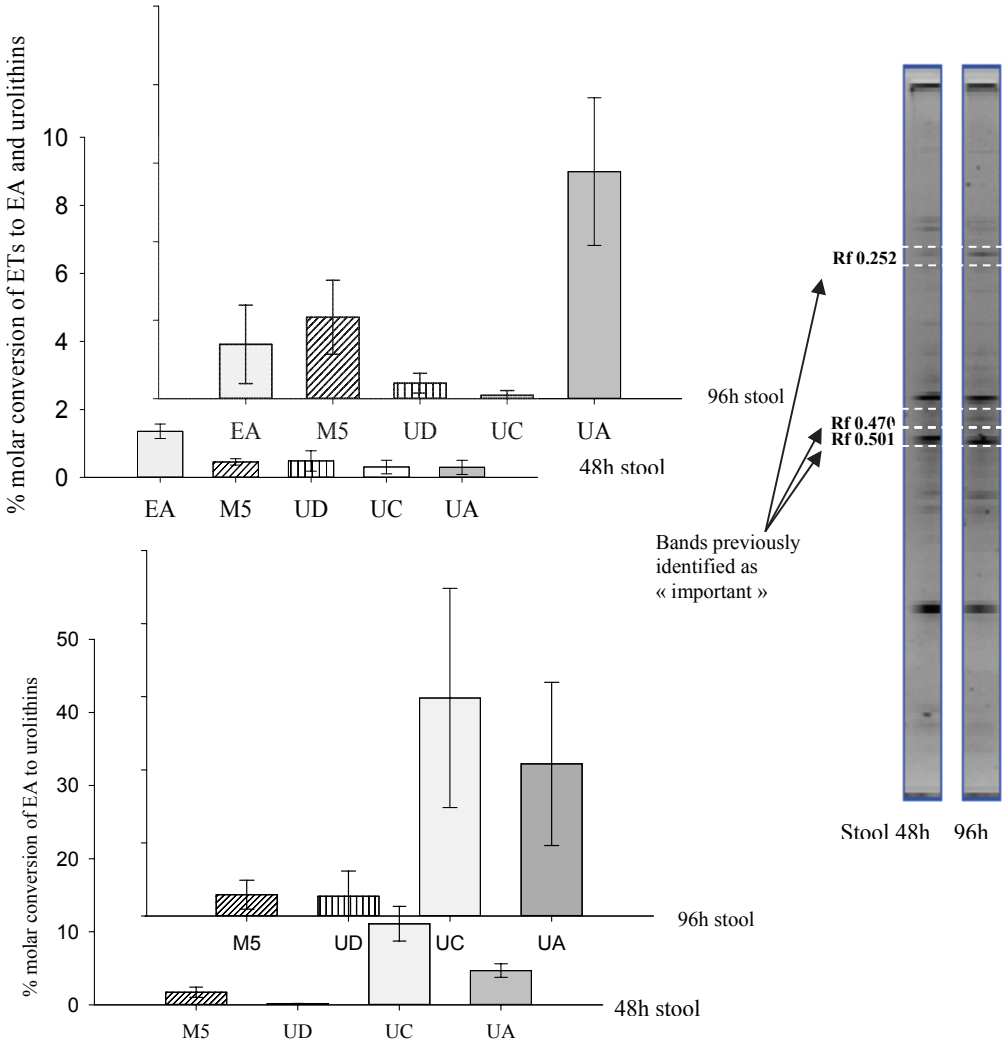

Figure 5.10 Percentage of molar conversion of EA or ETs (EA equ.) into primary ET-metabolites, when blackberry or ellagic acid (EA) are fermented 120 h with stools from one individual (individual G) collected at 48h and 96h after consumption of 250 ml of blackberry

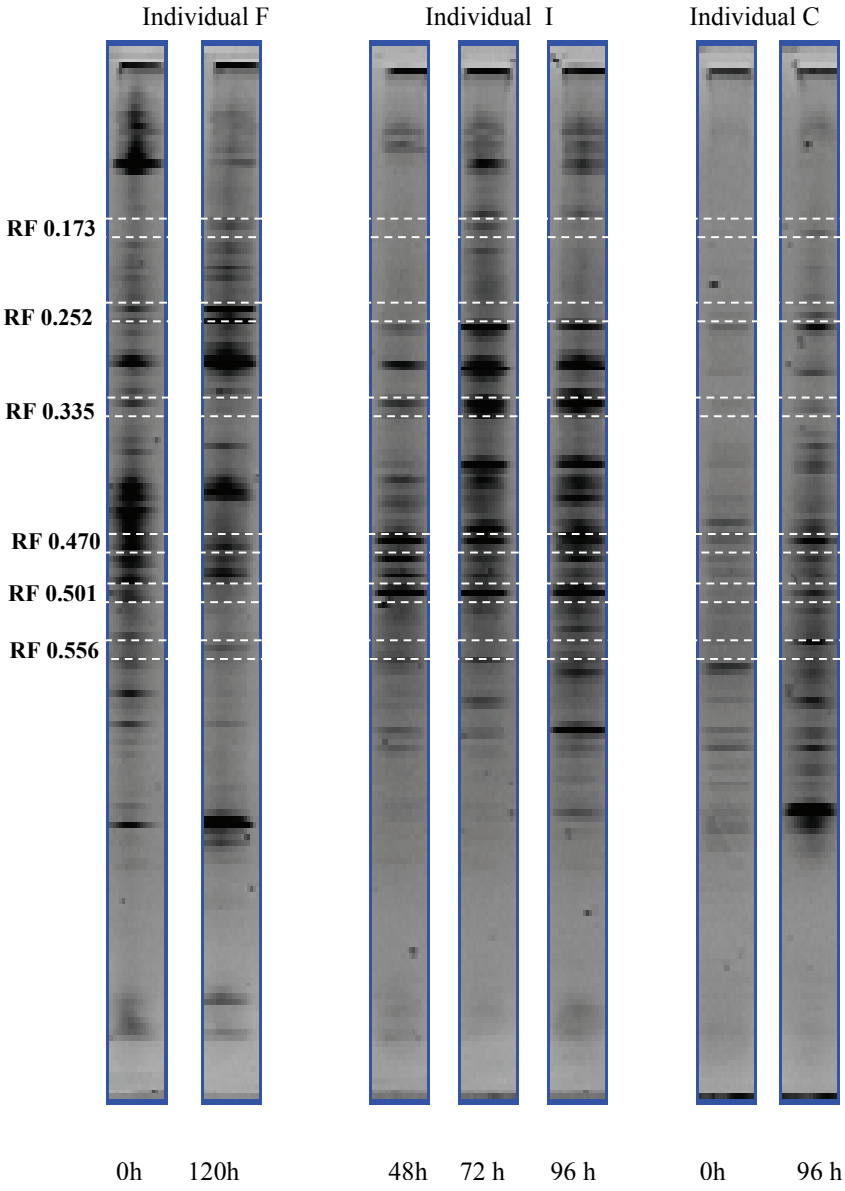

Figure 5.11 DGGE gel of PCR products of variable V3 region from 16S rDNA of fecal samples from 3 individuals (I , F and C) that donated fecal stool samples at 0, 48, 96 or 120 hours after ingesting one shot of 250-ml of BBJ.

6 CONCLUSIONS

According to these results, it was corroborated that the most important metabolite released from degradation of blackberry ellagitannins are UA and UB glucuronide derivatives. Nonetheless, a wide inter individual variability in the excretion of urolithins was observed while intra-variability along time was less pronounced. This work allows classifying for the first time volunteers into 3 classes. The first group comprises individuals that release urolithins at low concentrations, and then unlikely possibilities to obtaining any systemic health benefits from them. The second group corresponds to urolithins excreters that can be clustered in two subgroups: predominantly UA derivatives excreters and predominantly UB derivatives excreters. This status was found to be constant over time.

In vitro models of EA degradation by intestinal microflora under anaerobic conditions clearly showed the presence of ETs metabolites from the family dibenzopyranones, such as UA, three isomers of UC, two isomers of UD and UM5 confirmed the microbial origin of UM5, UD, UC and UA. This *in vitro* model of EA degradation is in accordance with the metabolic pathway that proposes the opening of lactone ring and subsequent dehydroxylation of UM5 to UD, UC and then to UA. However from clinical and *in vitro* models of bioconversion of ellagitannins from BBJ, the metabolic route appears to be different since intermediate metabolites such as UM5, UD, UC were hardly found. Although EA was easily released from blackberry ellagitannins, it was not clear how this EA released from the juice is converted into UA and UB.

From *in vitro* test urolithin B, UB was not found even using stools from predominant UB excreters. This leads to propose that UB could be the product of an interaction between gut microbiota and host. UA derivatives could be re-circulated in the bile facilitating the dehydroxylation of UA derivative to UB by enzymes or other inherent host factors that could play an important role in the UB production. However it is also possible that bacteria involved in UB production requires stricter conditions regarding total oxygen absence, redox potential, among others. Thus, probably under the conditions set up in the *in vitro* trials those bacteria were not able to grow. It can be state that UB require more complex conditions than UA, as UA can be released by most of urolithins excreters, and also from in

vitro test, conversely to UB which was found only from clinical test. Interactions between host excretory system and composition of gut microbiota were discussed as possible cause of presence of UB. Hepatic recirculation and additional deconjugation of UAG into UA and the further hydroxylation by cytochrome CYP450 could explain the predominant excretion of UB in some volunteers. Finally, thanks to molecular fingerprinting methods, such as PCR-DGGE, the screening of the gut microbiota ecology was carried out and by further multivariate statistical analysis, it was possible to observe a link between microbiota composition profile and ability to excrete urolithins. A group of microorganisms was correlated with the different cluster of urolithins excreters. Unfortunately, very little is known on gut bacteria, but most bacteria selected are linked to a healthy bowels confirming additional positive impact of ellagitannins in the gastro-intestinal tract.

Nonetheless, more works remain to do, and other research issues are now open. On the basis of our results, main research perspectives should focus on obtaining an innovative functional food from tropical highland blackberry. The significant role played by gut microbiota in the ETs' metabolic fate and the potentially important contributions of ET-microbial derivatives to health benefits are issues that must be taken into account. The high variability of gut bacteria ecosystems among humans (which results in different abilities to produce microbial derivatives) transposes into equally enormous inter-individual variability of physiological responses to dietary ETs. Therefore, in terms of dietary ETs, we demonstrated that we are not all equal. Some people can expect quick and effective systemic health benefits, but others will—eventually—receive only limited health benefits that would probably be confined to the GI tract. What, therefore, should be the recommended dietary strategy? How can the functional food industry tackle this challenge to provide a food that will better benefit all consumers?

At least four strategies can be considered: (1) provide consumers with the exogenous and appropriate microorganisms they lack, that is, with a new generation of probiotic bacteria that will establish themselves, at least temporarily, in the large intestine and convert dietary ETs and EA as they pass through the GI tract; (2) modify gut microflora ecosystems by promoting endogenous beneficial microorganisms that can metabolize dietary ETs and EA into urolithins; (3) design a food matrix that more

efficiently enhances urolithin production by providing microbiota with optimal substrates for sufficient time; and (4) execute *in vitro* what some people cannot do efficiently *in vivo*, for example, provide food that already contains urolithins.

In all cases, the first step is to confirm if the identified microorganisms, once isolated, are able to perform in vitro the bioconversion of ellagitannins into urolithin. Nonetheless, the bacteria we have identified in this work are not recognized as safe out of the gut where they can be involved in contamination. Consequently, these bacteria cannot be used as probiotic bacteria in dietary strategies to enhance the release of urolithins from ETs in the colon.

The second strategy is to promote endogenous gut-microbial ecosystems that more efficiently convert ETs. It raises the question of possibly transforming low-urolithin producers into high-urolithin producers by changing dietary habits or including prebiotics in the diet. As the notion of *urolithin-producer status* is relatively new, little information is available on how a human can change status by these means. We need to confirm if long exposure to ET-rich food could modify gut-microbiota ecology and induce the growth of urolithin-producing bacteria in human GI tracts. Our preliminary results on this subject tend to show that blackberry intake change gut ecology and enhance ability of stools to produce urolithins. Up to now, we don't know if some classical prebiotics could enhance the growth of the microorganisms that we have identified. Actually, they are apparently linked to healthy bowels, which could be eventually related to healthier diet. However, promoting transitory change of gut ecology could constitute a promising research topic for enhancing urolithin production, not only by affecting microbiota ecosystems but also by modulating metabolic pathways.

The third approach is to formulate a food matrix that improves the bioavailability of urolithins. Nothing is known about the dietary ingredients that would act synergistically with ETs and enhance urolithin excretion, but a variety of food ingredient could be tested. The interaction of ETs with other polyphenols, fats, sugars, proteins, should be investigated to get insights to design process and food products that enhance the formation of urolithins For example, it appears that pectin, a soluble fiber is substrate to at least two of the micro-

organisms highlighted, the *bacteroides spp* and *Faecalibacterium prausnitzii*. Therefore, enrichment with pectin of blackberry juice should be tested.

Modulation of intestinal transit time may also be an additional factor that could influences urolithin production. Transit time commands the period of interaction between substrate and microflora. Actually, the influence of food matrix and ET structure on urolithin excretion deserves more attention from researchers as better understanding of the mechanisms involved would be immediately useful for the food industry.

The fourth research approach concerns the *in vitro* production of urolithins, which could then be directly incorporated into food. This subject raises other issues such as the innocuity, pharmacokinetics, and stability of urolithins within the food matrix. Additionally, this strategy is more adapted to the development of dietary supplements and even drugs.

This description of research perspectives to better exploit dietary ETs highlights the main gaps to be addressed in the future. Indeed, research on ellagitannins is unbalanced, with emphasis being given more to the biological activities of ETs and their metabolites than to their bioavailability and or to the possibilities of modulating it in the design of relevant dietary strategies. Such emphasis has even been counterproductive to the better understanding of the potential health benefits of ETs in clinical studies.

We expect that results achieved in this project will contribute to the better understanding of ellagitannins metabolism and provide insights for future projects that must integrate diverse fields of the science, such as nutrition and biochemistry, microbiology, genetics; and, the food industry that ultimately could bring to consumers health prevention benefits associated to tropical highland blackberry juice and other ellagitannins ‘rich source.

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