UNIVERSIDADE FEDERAL DO RIO DE JANEIRO

IRIS BATISTA LEITE

POTENTIAL OF JABOTICABA POWDER CONSUMPTION TO MODIFY UROLITHIN EXCRETION AND METABOTYPE: A CLINICAL STUDY CONTROLLED BY PLACEBO

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Tese de Doutorado apresentada ao Programa de Pós-graduação em Nutrição do Instituto Josué de Castro, Universidade Federal do Rio de Janeiro, como requisito à obtenção do título de Doutor em Nutrição Humana

Orientadores:
Prof. Dra. Mariana Costa Monteiro
Prof. Dr. Daniel Perrone Moreira
Prof. Dra. Kim Ohanna Pimenta Inada

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Aprovado por:

Presidente, Profª. DSc. Mariana Costa Monteiro, INJC/UFRJ

Profª. DSc. Tatiana El-Bacha Porto, INJC/UFRJ

Profª. DSc. Eliane Lopes Rosado, INJC/UFRJ

Profª. DSc. Neuza Mariko Aymoto Hassimotto, FCS/USP

Profª. DSc. Mário Roberto Maróstica Junior, FEA/UNICAMP

Profª. DSc. José Luiz Alves Brito, PPGCN/UFPB

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RESUMO

Leite, Iris Batista. Potential of jabuticaba powder to modify urolithin excretion independent of weight in adults: a clinical study controlled by placebo. Rio de Janeiro, 2023. Tese (Doutorado em Ciências Nutricionais) – Instituto de Nutrição Josué de Castro, Universidade Federal do Rio de Janeiro, Rio de Janeiro, 2023.

A estratificação dos indivíduos em metabotipos em estudos clínicos torna-se cada vez mais importante para observar o efeito de uma intervenção alimentar com desfechos em saúde. A jaboticaba, uma *berry* brasileira, possui cerca de 40% a 50% de seu peso representado por casca e semente, que possui alto teor de compostos fenólicos como elagitaninos. Esse resíduo da jabuticaba já foi utilizado anteriormente para metabolização de elagitaninos na população brasileira, porém com altas doses, que não são compatíveis com o consumo habitual. O objetivo dessa tese foi caracterizar e avaliar a estabilidade dos compostos fenólicos da casca e semente de jaboticaba em pó (CSJP), e avaliar se o seu consumo por três semanas poderia modificar a caracterização inicial do metabotipo de urolitinas (A, B ou 0), assim como o grau de excreção de urolitinas. O pó de jaboticaba foi obtido após desidratação da casca e semente de jaboticaba em estufa de circulação forçada de ar (75 °C/ 22 h). Em seguida, o resíduo seco foi triturado, encapsulado em cápsula opaca e armazenado a 25 °C e a 5 °C por 57 dias. O teor de compostos fenólicos foi avaliado após 57 dias por HPLC-DAD. Para o estudo clínico, foram recrutados voluntários adultos (n = 59), de ambos os sexos, com índice de massa corporal (IMC) entre 18,5 e 40 kg/m^2. O estudo consistiu no consumo diário de quatro cápsulas contendo placebo (2 g de amido de milho) ou 4 cápsulas de CSJP (3 g do pó, contendo 35,2 mg de vescalagina + ácido elágico) por três períodos diferentes de três semanas, na seguinte ordem: 1) consumo de placebo; 2) consumo de JPSP; 3) consumo de placebo. Para verificar possíveis alterações nos metabotipos dos indivíduos e na quantidade de excreção de urolitinas, os voluntários ingeriram 3 g de CSJP logo antes do início de cada mensuração e após a última mensuração (urina 1, urina 2, urina 3 e urina 4). Amostras de urina (período entre 24 h e 36 h após a ingestão) foram coletadas antes e após todas as intervenções para análise do metabotipo e grau de excreção de urolitinas. Considerando todos os voluntários (n = 59), a distribuição dos metabotipos na urina 1 foi de 32,2% do metabotipo A (UM-A), 52,5% do metabotipo B (UM-B) e 15,3% do metabotipo 0 (UM-0), que diferiram dos já relatados na literatura em diferentes populações. Não foi encontrada diferença estatística entre os sexos quanto à distribuição de UM-A e UM-B. Considerando o IMC, os voluntários eutróficos apresentaram maior prevalência de UM-B (p

= 0,04) do que UM-A. Em contraste, os voluntários com sobrepeso/obesidade apresentaram uma distribuição semelhante de UM-A (43%) e UM-B (40%). Observou-se que com o aumento da idade houve aumento do percentual de UM-B e diminuição do percentual de UM-A. Após o consumo de CSJP, seis metabólitos foram identificados na urina: urolitina A 3/8-glucuronídeo, isourolitina A 3-glucuronídeo, isourolitina A 9- glucuronídeo, isourolitina A, urolitina B-glucuronídeo e urolitina B. As formas glucuronidadas foram as mais abundantes, representando 93-99% do total excretado em todas as etapas do estudo. Observou-se também uma grande variabilidade interindividual na excreção total de urolitinas (0,06 µmol a 15,39 µmol). Não foi observada diferença significativas quando os voluntários foram separados por sexo e % de gordura corporal. No entanto, quando os voluntários foram classificados de acordo com o IMC, observou-se que a intervenção com a CSJP levou a um aumento (p = 0,04) na excreção urinária de urolitinas nos voluntários eutróficos, enquanto para os voluntários com sobrepeso/obesidade essa alteração não foi observada. Portanto, foi possível observar que o consumo diário de CSJP foi capaz de aumentar a excreção de urolitinas após 3 semanas de exposição a uma fonte alimentar de elagitaninos em indivíduos eutróficos.

Palavras-chave: elagitaninos, metabotipos, peso adequado, obesidade

ABSTRACT

Leite, Iris Batista. Potential of jaboticaba powder to modify urolithin excretion independent of weight in adults: a clinical study controlled by placebo. Rio de Janeiro, 2023. Tese (Doutorado em Ciências Nutricionais) – Instituto de Nutrição Josué de Castro, Universidade Federal do Rio de Janeiro, Rio de Janeiro, 2023.

The stratification of individuals into metabotypes in clinical studies becomes increasingly important to observe the effect of a dietary intervention on health outcomes. Jaboticaba, a Brazilian berry, has about 40% to 50% of its weight represented by peel and seed, with a high content of phenolic compounds such as ellagitannins. This jaboticaba residue has previously been used to investigate ellagitannins metabolization in the Brazilian population but with high doses incompatible with regular consumption. The objective of this thesis was to characterize and evaluate the stability of the phenolic compounds in the powdered jaboticaba peel and seed (JPSP) and to evaluate whether their consumption for three weeks could modify the initial characterization of the urolithins metabotype (A, B, or 0), as well as the degree of excretion of urolithins. The jaboticaba powder was obtained after dehydration of the jaboticaba peel and seed in a forced air circulation oven (75 °C/22 h). Then, the dry residue was milled, encapsulated in an opaque capsule, and stored at 25 °C and 5 °C for 57 days. The content of phenolic compounds was evaluated after 57 days by HPLC-DAD. For the clinical study, volunteers (n = 59), of both sexes, with a body mass index (BMI) between 18.5 and 40 kg/m^2 were recruited. The study consisted of the daily consumption of four capsules containing placebo (2 g of corn starch) or four capsules of JPSP (3 g of powder, containing 35.2 mg of vescalagin + ellagic acid) for three different periods of three weeks in the following order: 1) placebo consumption; 2) JPSP consumption; 3) placebo consumption. To verify possible alterations in the individuals' metabotypes and the amount of urolithin excretion, the volunteers consumed 3 g of JPSP just before the beginning of each measurement and after the last measurement (urine 1, urine 2, urine 3, and urine 4). Urine samples (between 24 h and 36 h after ingestion) were collected before and after all interventions to analyze the metabotype and degree of urolithin excretion. Considering all volunteers (n = 59), the distribution of metabotypes in urine 1 was 32.2% of metabotype A (UM-A), 52.5% of metabotype B (UM-B), and 15.3% of metabotype 0, which differed from those already reported in the literature in different populations. No statistical difference was found between the sexes regarding the distribution of UM-A and UM-B. Considering BMI, volunteers with eutrophy had a higher

frequency of UM-B (p = 0.04) than UM-A. In contrast, overweight/obese volunteers had a similar distribution of UM-A (43%) and UM-B (40%). With increasing age, there was an increase in the percentage of UM-B and a decrease in the percentage of UM-A. After consumption of JPSP, six metabolites were identified in the urine: urolithin A 3/8-glucuronide, isourolithin A 3-glucuronide, isourolithin A 9-glucuronide, isourolithin A, urolithin B-glucuronide and urolithin B. The glucuronidated forms were the most abundant, representing 93-99% of the total excreted in all stages of the study. Interindividual variability was also observed in the total excretion of urolithins (0.06 µmol to 15.39 µmol). No significant differences were observed when the volunteers were separated by sex and % body fat. However, when the volunteers were classified according to BMI, it was observed that the intervention with the JPSP led to an increase (p = 0.04) in the urinary excretion of urolithins in volunteers with eutrophy. In contrast, for the overweight/obese volunteers, this alteration was not observed. Therefore, it was possible to observe that the daily consumption of JPSP increased the excretion of urolithins after three weeks of exposure to a dietary source of ellagitannins in individuals with eutrophy.

Keywords: ellagitannins; metabotypes; normoweight; obesity

RESULTING PUBLICATION FROM THIS PhD THESIS

1) Leite IB, Magalhães CD, Monteiro M, Fialho E. (2021). Addition of Honey to an Apple and Passion Fruit Mixed Beverage Improves Its Phenolic Compound Profile. **Foods**,10(7):1525. https://doi.org/10.3390/foods10071525
2) Inada, K. O. P., Leite, I. B., Martins, A. B. N., Fialho, E., Tomás-Barberán, F. A., Perrone, D., & Monteiro, M. (2021). Jaboticaba berry: A comprehensive review on its polyphenol composition, health effects, metabolism, and the development of food products. **Food Research International,** 147, 110518. https://doi.org/10.1016/j.foodres.2021.110518

LIST OF ABBREVIATIONS

ADME: absorption, distribution, metabolism, and excretion

BF: body fat

BMI: Body mass index

Cmax: maximum plasmatic concentration

COST-Positive: Cooperation in Science and Technology-Positive

CYP: cytochrome P450

DW: dry weight

GAE: gallic acid equivalent

HHDP: hexahydroxydiphenoyl

HPLC: High-Performance Liquid Chromatography

IsoUro-A 3-glu: isourolithin A 3-glucuronide

IsoUro-A 9-glu: isourolithin A 9-glucuronide

IsoUro-A: isourolithin A

JPSP: jaboticaba peel and seed powder

ODMA: *O*-desmethylangolesin

UGT: glucuronosyltransferase

UM-0: metabotype 0

UM-A: metabotype A

UM-B: metabotype B

Uro-A 3/8-glu: urolithin A-3/8-glucuronide

Uro-A: urolithin A

Uro-B: urolithin B

Uro-B-glu: urolithin B-glucuronide

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SUMMARY

Thesis introduction

The various beneficial health effects associated with the consumption of fruits and vegetables are due, in part, to the presence of phenolic compounds. However, some clinical trials have failed to demonstrate the beneficial health effects of some phenolic compound's sources. These results can be justified due to differences in the composition of the intestinal microbiota among individuals, which associates with intestinal inflammation and health. Intestinal bacteria promote the metabolization of phenolic compounds, producing several metabolites which may present different biological activities. Therefore, the stratification of individuals into metabotypes becomes necessary to better understand the health effects associated with foods rich in phenolic compounds. Regarding the metabolism of ellagitannins, the classification of individuals according to their metabotype is described in the literature, namely: metabotype A (urolithin A producers), metabotype B (urolithin B, isourolithin-A and urolithin A producers) and metabotype 0 (non-urolithin producers). Among the sources of ellagitannins, jaboticaba, a Brazilian berry, stands out, and it has been previously used to characterize the Brazilian population about the urolithin metabotype after intake of high amounts of this food source. The present study is the first to carry out this characterization following the intake of low amounts of ellagitannins and to assess whether chronic consumption affects urolithin excretion following jaboticaba intake.

Therefore, the general objective of this thesis was to investigate the effect of consumption during 3 weeks of jaboticaba peel and seed powder on the metabolism of their ellagitannins in normoweight and overweight/obese adults. Additionally, we investigate the stability of phenolic compounds in jaboticaba peel and seed powder stored at different conditions for 57 days; stratify the studied population according to the ellagitannin metabolization profile (metabotype A, metabotype B, and metabotype 0); and investigate the effect of jaboticaba peel and seed powder and/or placebo consumption on the modification of the ellagitannin metabolization profile and excretion.

LITERATURE REVIEW

1. Phenolic compounds

Phenolic compounds, found in vegetables, fruits, and seeds, are secondary metabolites produced in response to stress (Eseberri et al., 2022; Morand et al., 2020). They present benefits to plants, such as contribution to their pigmentation, protection against ultraviolet light, and influence growth, and structure, among others (González-Sarrías et al., 2020; Santos-Sánchez et al., 2019). There are two metabolic pathways in which these compounds can be formed: shikimic acid and malonic acid. In plants, the primary means of biosynthesis of phenolic compounds occurs through the shikimic acid pathway, which consists of seven steps beginning with an aldol-type condensation of phosphoenolpyruvic acid from the glycolytic pathway to chorismic acid, its final product (**Figure 1**).

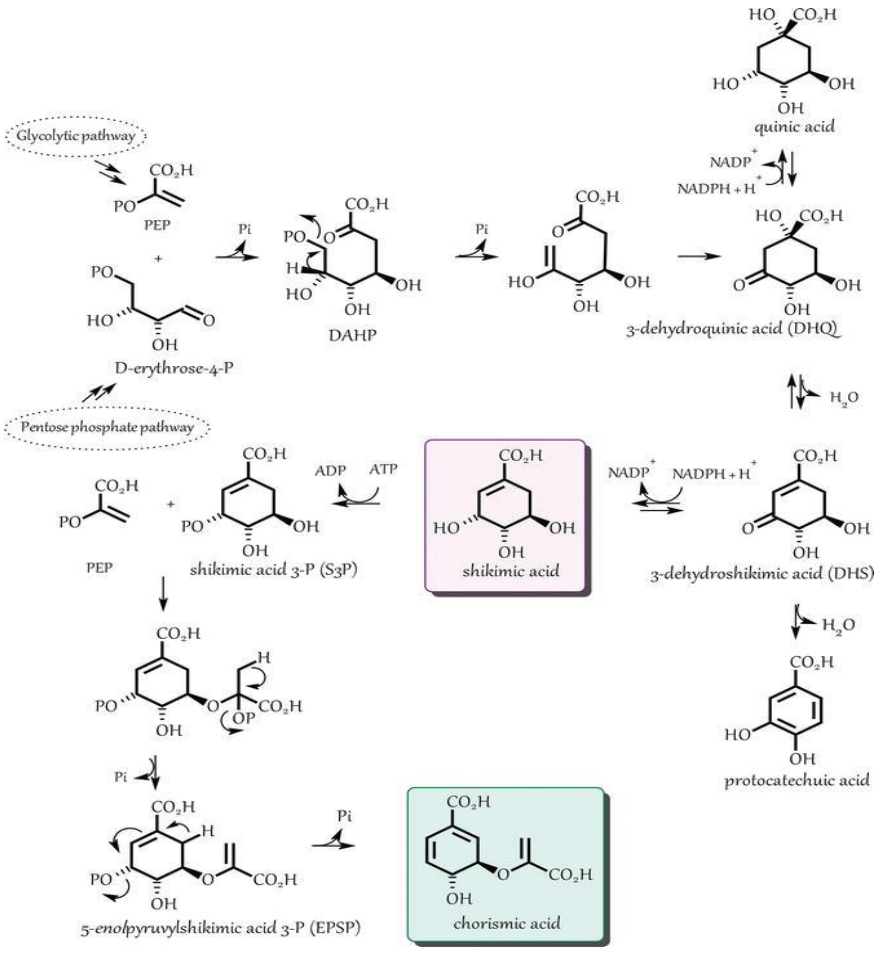

Figure 1. Biosynthesis of phenolic compounds from the shikimic acid pathway (Santos-Sánchez et al., 2019). PEP: Phosphoenolpyruvate; DAHP: 3-Deoxy-D-arabino-heptulosonic acid 7-phosphate; Pi: Pi, phosphate; NAD+, oxidized nicotinamide adenine dinucleotide; NADPH, reduced nicotinamide adenine dinucleotide phosphate.

Phenolic compounds are a group of more than 8,000 substances with different structures characterized by at least one aromatic ring with one or more attached hydroxyl groups. In plants, phenolic compounds are mainly found as conjugates (as opposed to aglycones), being most commonly linked with one or more sugar residues, mainly glucose. They are divided into two basic structures, flavonoids and non-flavonoids (Del Rio et al., 2013; Manach et al., 2004).

Flavonoids are comprised of 15 carbons, usually with two aromatic rings, connected by three linear carbons that form a heterocyclic pyran ring containing an oxygen atom and are subdivided into several groups (flavonols, flavanones, isoflavones, flavan-3-ols, and anthocyanidins). Non-flavonoids, on the other hand, are composed of one or two aromatic rings. They can be classified as hydroxybenzoic acids, hydroxycinnamic acids, hydrolyzable tannins with one or two aromatic rings, and stilbenes (**Figure 2**) (Morand et al., 2020; Kay et al., 2020).

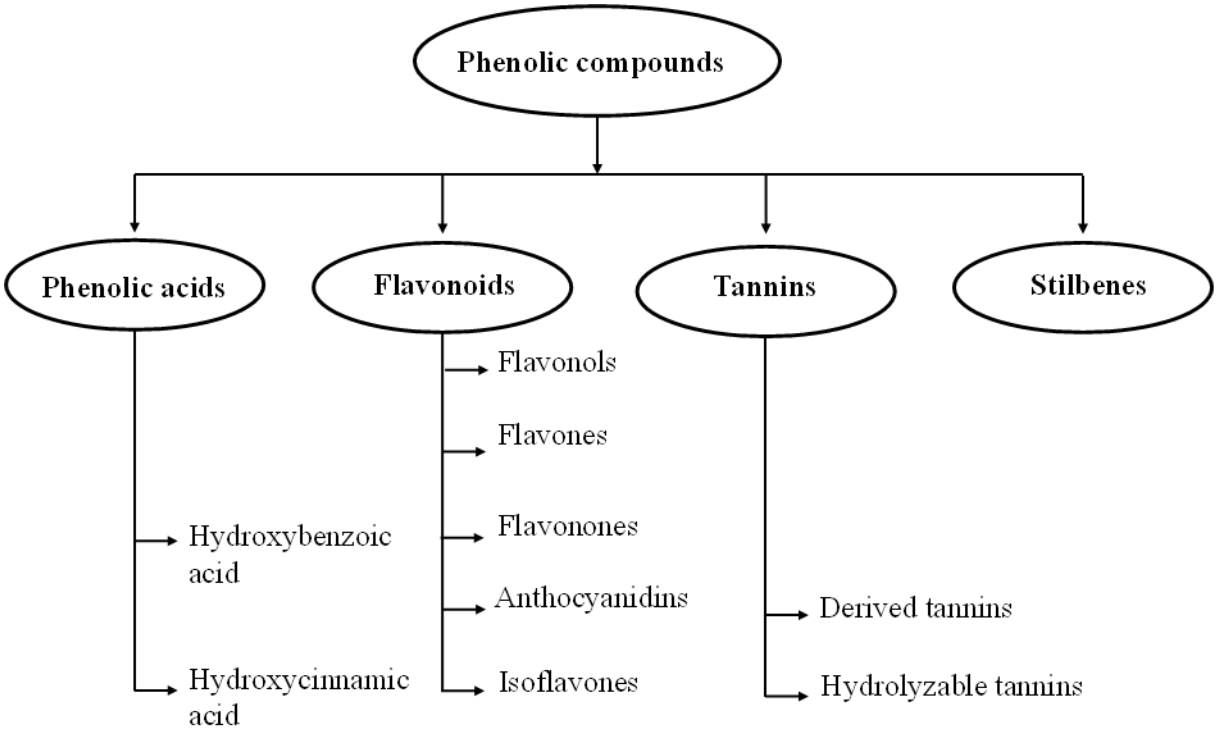

Figure 2. Distribution of phenolic compounds in classes and subclasses (adapted from Alara et al., 2021).

Phenolic compounds are found in fruits, vegetables, seeds, cereals, and beverages, such as coffee and tea, and their daily intake can reach values greater than 2 g/day and may differ according to one's eating habits (Morand et al., 2020; Zamora-Ros et al., 2016). In addition to dietary habits, the content of phenolic compounds in foods varies due to

environmental and processing factors such as temperature, water supply, exposure to UV light, storage, and culinary preparation (Gudžinskaitė et al., 2020; D'Archivio et al., 2007; Manach et al., 2004).

The investigation of the health effects associated with the consumption of these compounds usually takes place primarily through *in vitro* and animals' studies, providing preclinical evidence of their health benefits, such as maintenance of cardiovascular health and prevention of non-communicable diseases, such as diabetes and cancer (Fraga et al., 2019; Zamora-Ros et al., 2016). The observed effects of phenolic compounds derived from *in vivo* studies depend on their bioaccessibility - the amount available for absorption after digestion – and bioavailability- the proportion available after metabolization (Zamora-Ros et al., 2016; Manach et al., 2004). After this process, systemic availability typically varies in the nM and μM range (Del Rio et al., 2013; Manach et al., 2004). More information on the metabolization of compounds will be provided in section 4.

2. Jaboticaba

Jaboticaba, which may be called a Brazilian berry, is found extensively in Southeast South America (Oliveira et al., 2019). The three most abundant species in Brazil are *Myrciaria jaboticaba (Vell.) Berg* ("Sabará" jabuticaba), *M. trunciflora O. Berg* ("Cabinho" jabuticaba) and *Myrciaria cauliflora (Mart.) O. Berg* (popularly known as "Paulista", "Ponhema," or "Assu" jabuticaba). Jaboticaba belongs to the *Myrtaceae* family, also called Plinia (Citadin et al., 2010). In Brazil, it blooms between March and October; the states with the highest production are Goiás, São Paulo, and Minas Gerais (Oliveira et al., 2019; IBGE, 2017; Citadin et al., 2010)**.** Due to the many beneficial health effects reported in *in vitro*, animal and human studies, like antioxidant, antiproliferative, anti-inflammatory, antimicrobial, and cardioprotective properties, it has recently been called a superfruit (Inada et al., 2021; Chang et al., 2019).

Jaboticaba fruits are small berries 3–4 cm in diameter, and it contains a gelatinous sweet pulp with one to four small seeds. Jaboticaba peel is dark purple or black after ripening (**Figure 3**), and its astringency is caused by the phenolics present in the peel, particularly ellagitannins (Oliveira et al., 2019). Its high sugar and water contents, mainly from the pulp, make this fruit highly perishable. This factor makes its commercialization *in natura* difficult,

which justifies its use in preparations such as juices and jellies (Albuquerque et al., 2020; Chang et al., 2019).

Figure 3. Jaboticaba tree with jaboticaba ripped fruit (Fonte: Ana Beatriz Neves).

The peel and seed correspond to about 50% of the fruit and are considered waste in the manufacture of products (Morales et al., 2016). This residue, which is usually discarded, would contribute to a more sustainable and economical use of the fruit (Ravindran & Jaiswal, 2016).

Jaboticaba carbohydrate content is higher than grumixama (*Eugenia brasiliensis*), cereja do Rio Grande (*Eugenia involucrata*), and Brazilian purple fruits. Part of the carbohydrate comes from its fiber content (3.47 to 3.88 g/100 g, on a fresh weight basis) (Schulz et al., 2020). Jaboticaba is a source of vitamin C, copper, iron, and beta-carotene, and, most importantly, a rich in phenolic compounds (Schulz et al., 2020; Inada et al., 2015). Among the various phenolic compounds, anthocyanins and ellagitannins stand out, which are mostly concentrated in the peel and seed (Inada et al., 2021; Pereira et al., 2017; Inada et al., 2015).

2.1 Phenolic compounds in jaboticaba

More than 80 types of phenolic compounds have already been described in the jaboticaba species' pulp, peel, and seeds (Inada et al., 2021). These include anthocyanins, hydroxybenzoic acid derivatives (ellagitannins, gallotannins, and ellagic acid derivatives), hydroxycinnamic acids, flavonols, flavanols, flavanones, and flavones (Inada et al., 2021). The total content of phenolic compounds can reach values up to 6,000 mg of gallic acid equivalents (GAE)/100 g in ripe fruit (expressed on dry weight basis). Thus, jaboticaba could be characterized as a Brazilian fruit rich in phenolic compounds according to De Souza et al. (2012), which classifies fruits into low (<100 mg GAE/100 g), medium (100–500 mg GAE/100g), and high (>500 mg GAE/100 g) content of phenolic compounds (Schulz et al., 2020).

Anthocyanins are the class of compounds with the highest content in jaboticaba, and it is responsible for the purple color of the peel. Its content can reach a value higher than 3 mg/100 g in the peel, of which cyanidin-3-*O*-glucoside and delphinidin-3-*O*-glucoside are predominant (Inada et al., 2021). Another class of compounds found mainly in the peel and seeds are hydrolyzable gallotannins and ellagitannins. Among the latter, vescalagin, castalagin, and peduncalagin are found in higher contents, reaching values of approximately 3 mg/100 g in the ripped fruit (Inada et al., 2020).

Most studies with foods evaluated the soluble phenolics profile, corresponding to compounds present in vacuoles, and can be extracted by aqueous solvents (Pérez-Jiménez et al., 2013). However, many phenolic compounds are linked to food matrix components, such as pectin, cellulose, and protein structures. In this case, phenolic compounds are usually extracted by alkaline hydrolysis, followed by acid hydrolysis, which releases the maximum number of compounds linked to the food matrix. Alkaline hydrolysis cleaves ester and ether bonds, while acid hydrolysis mostly breaks down glycosidic bonds (Shahidi & Yeo, 2016). Therefore, analyzing soluble and insoluble food compartments is important to understand the whole picture of food phenolic compounds (Shahidi & Yeo, 2016).

Ellagitannins' contents can be underestimated mainly due to the extraction method used. Ellagitannins are covalently linked to the food matrix, so alkaline and acid hydrolysis extraction is required to release them (García-Villalba et al., 2015). In some cases, this extraction leads to hydrolysis and depolymerization, releasing gallic and ellagic acids, which are used as proxies of ellagitannins content in food, especially for those without commercial

standards (Montes-Ávila et al., 2017; García-Villalba et al., 2015). The content of anthocyanin and ellagitannins can also vary according to the jaboticaba species - *Myrciaria jaboticaba* peel has higher contents of anthocyanins. In contrast, Myrciaria *trunciflora* peel has higher contents of hydrolyzable tannins (Quatrin et al., 2019).

2.1.1 Ellagitannins in jaboticaba

Ellagitannins are a type of tannins that are mainly present in the peel, stems, seeds, and sprouts of some foods such as fruits (grapes, blackberries, blueberries), oilseeds (walnuts, almonds, cashew nuts), legumes (beans, peas, lentils), and cereals (rice, buckwheat) (Das et al., 2020; Bule et al., 2020; Smeriglio et al., 2017). They belong to the non-flavonoid class and are divided into proanthocyanidins, hydrolyzable tannins, and condensed tannins. Tannins were first described as anti-nutritional compounds that must be removed during food processing (Villalba et al., 2019). However, current studies indicate that tannins present numerous biological activities, including anti-inflammatory, antioxidant, and antimicrobial activity (Törrönen, 2009; Cheng et al., 2020)

Even though ellagitannins are present in many foods, their consumption is relatively low because their food content is low and mainly concentrated in the peels, which are often discarded (Landete, 2011). Ellagitannins are located in the vacuoles and cytoplasm of plant cells and belong to the group of hydrolyzable tannins and are more commonly found in plants than gallotannins (Banc et al., 2023; Smeriglio et al., 2017). By forming complexes with proteins and polysaccharides, ellagitannins protect plants against attacks by animals and bacteria (Quideau & Feldman, 1996).

In nature, more than 1,000 ellagitannins have been described. Ellagitannins are esters of hexahydroxydiphenic acid (HHDP) with a D-glucose carbohydrate moiety, complex structures with high molecular weights of up to 4000 Daltons, and can be divided into monomeric and polymeric (Villalba et al., 2019; Yamada et al., 2018; Törrönen, 2009). Monomeric ellagitannins have one glucose nucleus (e.g., nufarin A, geraniin, tellimagrandin II, punicalagin, eugeniin, davidiin, casuaritin, and corilagin). Polymeric ellagitannins present two or more joint units of monomeric ellagitannins (nufarin E, nufarin C, agrimoniin and hyrtelin A) and glycosidic ellagitannin C (vescalagin, castalagin, stachyurin and casuarinin) (Smeriglio et al., 2017; Törrönen, 2009). The most commonly ingested by humans are punicalagin

(pomegranate), peduncalagin (walnuts), and sanguiin (strawberry and raspberry) (Larrosa et al., 2010).

The chemical structure of ellagitannins significantly influences its antioxidant activity, increasing according to the degree of hydroxylation and decreasing in the presence of a glycoside moiety (Landete, 2011). Due to the complexity of ellagitannins molecules, they can undergo isomerization, oligomerization, and acidic or basic hydrolysis reactions producing hexahydroxydiphenic acid, which is then spontaneously converted to ellagic acid (Villalba et al., 2019; Garrido & Borges, 2013; Törrönen et al., 2009). This susceptibility to hydrolysis is important for the health effects associated with ellagic acid consumption as anti-inflammatory, cardioprotective, and hepatoprotective (Sharifi-Rad et al., 2022; Klimczak & Król, 2010).

In addition to the foods mentioned above, some Brazilian fruits are sources of ellagitannins, such as jaboticaba (3.11 g of total EA/kg), grumixama (*Eugenia brasiliensi)* (2.70 g of total EA/kg), and cambuci (*Campomanesia phaea*) (2.67 g of total EA/kg). Their ellagitannin contents were shown to be higher than in camu-camu (*Amazonian fruit*) (0.59 g of total EA/kg) and Surinam acerola (*Brazilian acerola*) (0.96 g of total EA/kg) (Abe et al., 2012). In jaboticaba, 33 ellagitannins and their isomers have already been reported in the literature, especially vescalagin (740 to 1,805 mg/100 g, dw), castalagin (507 to 898 mg/100 g, dw) and peduncalagin (21 to 452 mg/100 g, dw) (Inada et al., 2021; Pimenta Inada et al., 2020; Quatrin et al., 2019; Pereira et al., 2017; Inada et al., 2015). The ellagitannin most recently found in *M. cauliflora* was cauliflorin (Pereira et al., 2017). The chemical structures of the major ellagitannins reported in jaboticaba are shown in **Figure 4**.

Among the jabuticaba species, *M. cauliflora* and *M. jaboticaba* are the most used in characterization and *in vivo* studies (Inada et al., 2021). Pereira et al. (2017) found that the content of vescalagin+castalagin in *M. cauliflora* was 81.1 mg/100 g of fruit peel powder, while Quatrin et al. (2019) found 15.3 mg/100 g of fruit peel powder in *M. jaboticaba* and 60.8 mg/100 g of fruit peel powder in *M. trunciflora*.

Some ellagitannins are found only in jaboticaba, such as sanguiin H-10 isomers and sanguiin H-6 (**Figure 5**), and they are found in high contents (1.9- 175 mg/ 100 g dw), especially in seeds (Inada et al., 2021). The contents of pedunculagin, castalagin, and vescalagin were higher in seeds. Cauliflorin, on the other hand, had a higher content in peels compared to the pulp, and it was not found in the seed, which is the part of the plant that suffers greater influence in terms of ellagitannins composition during fruit maturation (Pereira et al., 2017).

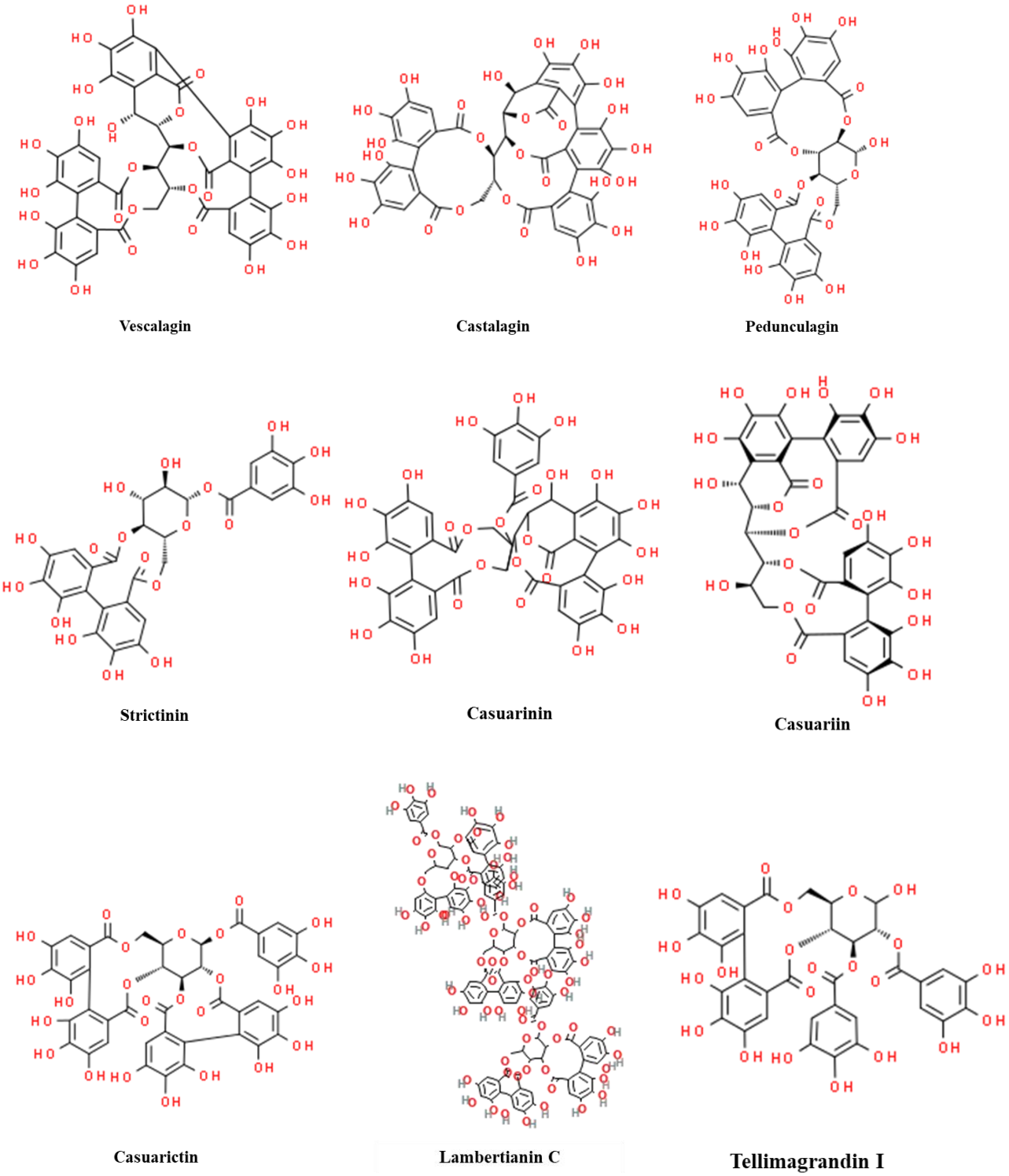

Figure 4. Structures of ellagitannins present in jaboticaba peel and seed (chemspider.com).

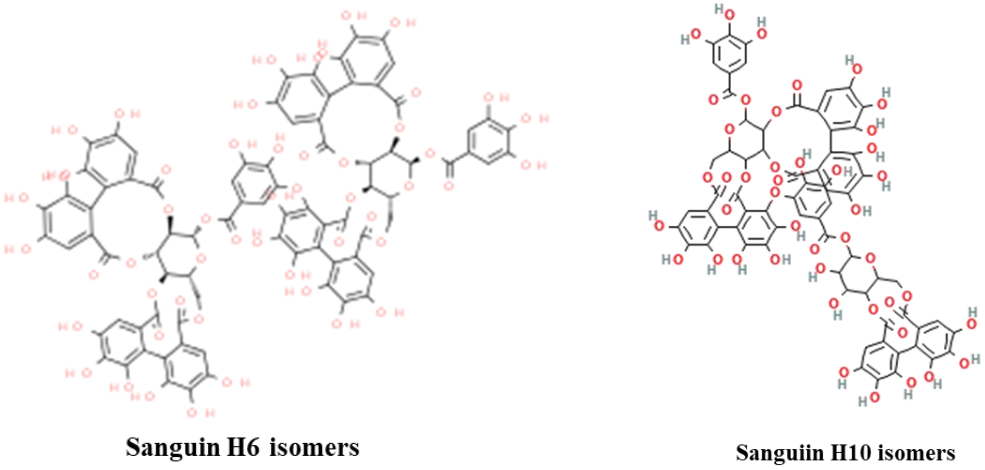

Figure 5. Structures of ellagitannins only present in jaboticaba peel and seed (chemspider.com).

In studies evaluating the health effects of jaboticaba consumption, ellagitannins are identified as one of the possible agents responsible for its antioxidant (Albuquerque et al., 2020; Plaza et al., 2016), chemopreventive and antimicrobial effects (Albuquerque et al., 2020). The antimicrobial properties of ellagitannins are explained by their ability to penetrate the phospholipid layer of animal cells due to their hydrophobicity. Once within cell membranes, ellagitannins inhibit membrane synthesis and trigger cell lysis by iron chelation (Farha et al., 2020; Machado et al., 2018).

Due to their large structures and the great diversity of ellagitannins isomers/types, they have low bioavailability and are bioconverted into lower molecular weight compounds by the gut microbiota. Thus, studies in humans have shown that the beneficial health effects associated with the consumption of food rich in ellagitannins are caused mainly by urolithins, the microbial metabolites of ellagitannins (Tow et al., 2022; Espín et al., 2013; Cardoso et al., 2013).

Another way of evaluating ellagitannins in foods is to quantify the content of ellagic acid resulting from the extraction of insoluble compounds of the food matrix by alkaline and acid hydrolysis. This occurs because ellagitannins are hydrolyzable compounds (Sharifi-Rad et al., 2022; Montes-Avila et al., 2017), and the acid condition promotes their hydrolysis and depolymerization, releasing mainly gallic acid and/or hexahydroxydiphenic acid, which in turn undergoes lactonization to ellagic acid (**Figure 6**) (Macierzyński et al., 2020). Ellagic acid can also be produced by enzymatic hydrolysis of ellagitannins in foods by tannases (tannin acyl hydrolase) and ellagitannins acyl hydrolases (ellagitannases) produced by *Aspergillus niger* (Dai et al., 2020; Ascacio-Valdés et al., 2014).

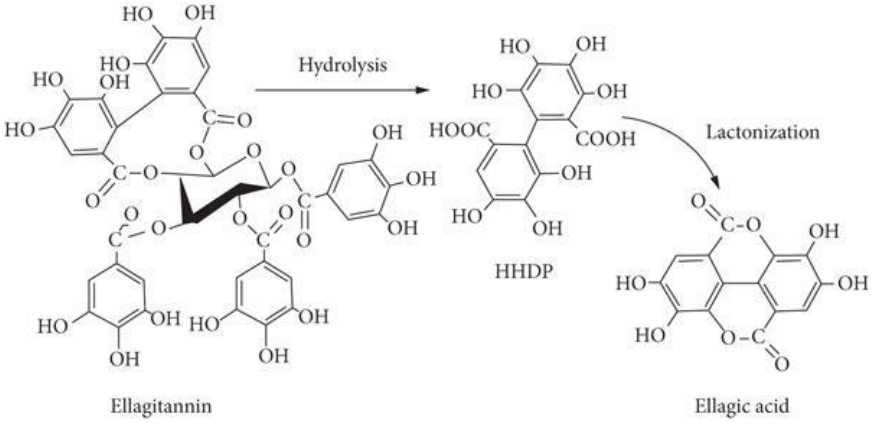

Figure 6. Formation of ellagic acid upon microbial hydrolysis of ellagitannin. HHDP: hexahydroxydiphenic acid (Ascacio-Valdés et al., 2014).

3. Metabolism of phenolic compounds

The dietary consumption of phenolic compounds can reach amounts as high as 2 g/day. However, some studies have demonstrated the importance of considering not only the amount but also the entire process of metabolization and absorption of these compounds (Gutiérrez-Díaz et al., 2021; Arranz et al., 2010). The phenolic compounds naturally present in foods usually have low bioavailability, but only 5% are absorbed. Moreover, there is a very high variability among individuals regarding the metabolism of phenolic compounds. Several factors are associated with this variability, which includes genetics, age, sex, body composition, gut transit, and gut microbiota composition, as they may modulate absorption, distribution, metabolism, and excretion (ADME) of phenolic compounds (Landberg et al., 2019; Manach et al., 2005).

The interaction among the factors that regulate ADME of phenolic compounds contributes to the difficulty in establishing causality and dose-effect response relationships in intervention studies that address the effects of the consumption of phenolic compounds in food on the reduction of cardiovascular risks (Manach et al., 2017; Milenkovic et al., 2017; Landberg et al., 2019). The absorption and bioavailability of polyphenols are largely influenced by the structures of these compounds, as mentioned above (Domínguez-Avila et al., 2017). For instance, only aglycones and, to a lesser extent, some glycosides can be absorbed directly by the intestinal mucosa (Sallam et al., 2021; Walle et al., 2005).

Gastrointestinal tract permeability is selective and controlled. It is suggested that only more lipophilic and low molecular weight phenolic compounds (for example, gallic acid) can

pass through claudins in the tight junctions of the intestinal epithelium due to the high electrical resistance that these proteins exert (Domínguez-Avila et al., 2017). Phenolic acids can be absorbed in the small intestine and liver and less expressively by passive diffusion or active transport in the stomach (Hussain et al., 2019; Del Rio et al., 2013; Selma et al., 2009; Manach et al., 2005). In the small intestine there are two potential mechanisms: the action of lactase-phlorizin hydrolase that deglycosylates phenolic groups or by cytosolic β-glucosidases where the glycosides are cleaved after being transported by the sodium-dependent glucose transporter 1 (SGLT1), both present in the enterocyte membrane stimulating the hydrolysis of glycosylated forms into aglycones that can enter cells by passive diffusion **(Figure 7)** (Mosele et al., 2015; Bohn, 2014; Del Rio et al., 2013).

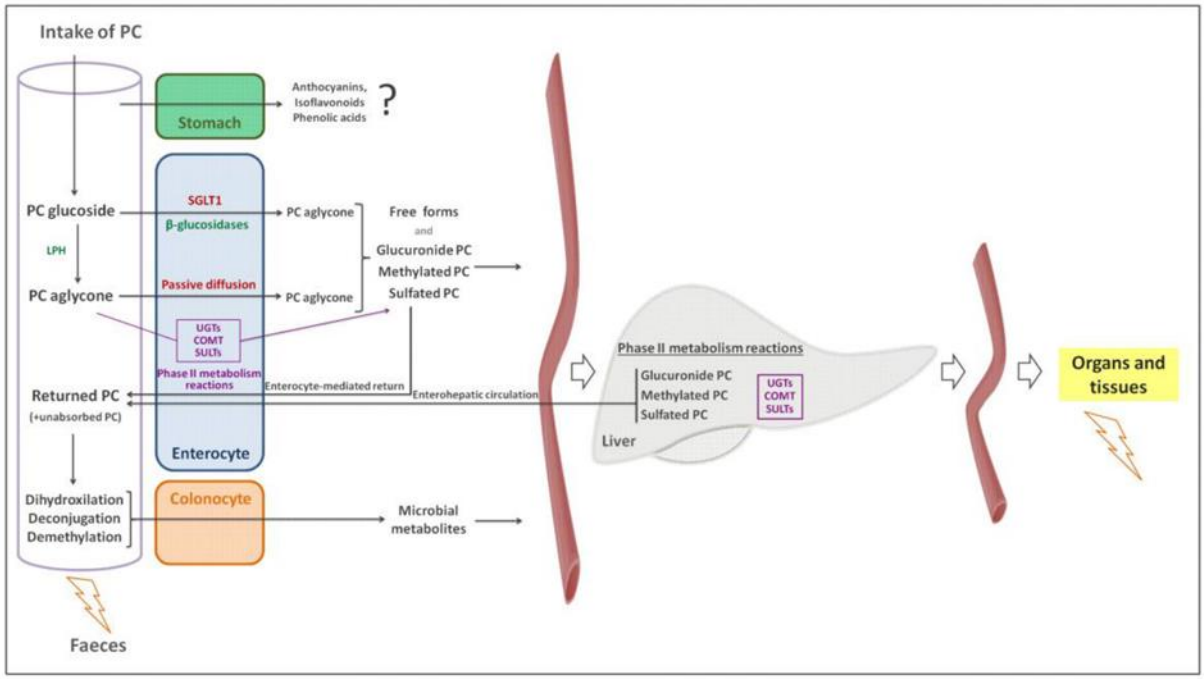

Figure 7. Cleavage mechanisms of glycosylated forms into aglycones (Eseberri et al., 2022). PC: phenolic compound; LPH: lactase-phlorizin hydrolase; SGLT1: sodium-dependent glucose transporter 1; UGTs: uridine 5-diphosphate glucuronosyltransferases; COMT: methylation by catechol-O-methyltransferase; SULTs: sulphotransferases.

In the small intestine, aglycones pass through the enterocyte via the basolateral membrane or exit the enterocyte via the apical membrane to return to the intestinal lumen. Metabolites passing through the basolateral membrane enter the portal vein to the liver, undergoing phase I and II metabolisms. In phase I metabolism, compounds are oxidized and demethylated by CYP monooxygenases in the liver, and in phase II, the metabolites from the

previous phase are subjected to glucuronidation, methylation, and sulfation (Hodek et al., 2002). Phase II reactions serve to facilitate biliary and urinary excretions by increasing hydrophilicity of parent compounds (Hussain et al., 2019; Hamauzu, 2018; Ozdal et al., 2016; Wu et al., 2011; Liu & Hu, 2007).

After modifications in the liver, the conjugated metabolites can follow two pathways: enter the systemic circulation and reach the target tissues or return to the intestine through the enterohepatic circulation. In the colon, they undergo the action of the intestinal microbiota (deglycosylation, dehydroxylation, demethylation, deconjugation, epimerization, ring fission, hydrolysis, and chain shortening), being part of the metabolites eliminated in the feces (Duarte et al., 2021; Possemiers et al., 2011; Manach et al., 2004). Upon reaching the liver and kidneys, they are excreted in the urine (Seyed Hameed et al., 2020).

The transformations by gut microbiota are different depending on the phenolic structure, degree of polymerization, and spatial configuration, so the conversion of aglycone forms into gut metabolites by fecal microbial enzymes (R-rhamnosidase, β-glucosidase, and β-glucuronidase) (Cortés-Martín et al., 2020a; Hamauzu, 2018; Selma et al., 2009). These metabolites tend to pass cell barriers more efficiently and to be more potent in terms of bioactivity compared to their parent compounds (Luca et al., 2020; Espín et al., 2017; González-Sarrías et al., 2017a).

The metabolites generated by the interaction of PC with the healthy intestinal microbiota (probiotics) fit the definition of a postbiotics, which is "any factor resulting from the metabolic activity of a probiotic or any molecule released capable of conferring beneficial effects to the host directly or indirectly" helping to maintain intestinal health and consequent host health (Malashree et al., 2019; Tsilingiri & Rescigno, 2013). Even though the mechanisms are not yet fully elucidated, there has been remarkable progress in the investigation of these metabolites and the action of these postbiotics, highlighting the derivatives of isoflavones, ET, and lignans that have specificity and individual variability in their production (Cortés-Martín et al., 2020a; Aguilar-Toalá et al., 2018).

In addition, there is a bidirectional relationship between gut microbiota and gut metabolites. The intestinal microbiota is involved in the breakdown of the food matrix and bioaccessibility of the compound. It can be influenced by the presence of this metabolite, changing the composition and functionality of the intestinal microbiome, thus facilitating absorption by modulating the intestinal barrier (Landberg et al., 2019).

4. Metabolism of ellagitannins

Ellagitannins are not absorbed in their natural form found in food due to their high molecular weight and polarity (Villalba et al., 2019; Quideau et al., 2011). When passing through the stomach and small intestine, these polyphenols suffer acid and base hydrolysis, respectively, and can release free ellagic acid (Villalba et al., 2019). These free ellagic acid have low bioavailability reaching the large intestine in almost intact form, where they are metabolized by the gut microbiota (Villalba et al., 2019; McDougall et al., 2014; Giampieri et al., 2012).

Its maximum plasma concentration (C_{max}) is highly variable and depends on ellagitannins and free ellagic acid contents. González-Sarrías et al. (2015) administered two pomegranate extracts to humans with low and high amounts of free ellagic acid. The intake of the pomegranate extract containing about 290 mg of ellagitannins plus 25 mg of free ellagic acid resulted in a C_{max} between 15 and 360 nM of ellagic acid. In contrast, administering about 130 mg of ellagitannins plus 524 mg of free ellagic acid resulted in a C_{max} from 12 to 193 nM. This work demonstrated that increasing the amount of free ellagic acid being consumed does not lead to higher plasma concentrations, probably due to its conversion to urolithins by the gut microbiota, as already mentioned. Urolithins metabolites are more readily absorbed than ellagic acid, highlighting the importance of studies addressing the mechanisms involved in urolithin metabolism.

Intestinal bacteria can hydrolyze the ester linkages of ellagitannins unaffected by acid and base hydrolysis. These ellagitannins can produce ellagic acid and glucose by tannin acyl hydrolase activity. Evidence from in vitro studies shows that complex ellagitannins such as punicalagin and peduncalagin may undergo hydrolysis reactions by microbial tannases. Both gallic acid and ellagic acid can undergo decarboxylation and dehydroxylation, and the latter can be further converted to nasutins, after the removal of two hydroxyl groups (**Figure 8**) (Tomás-Barberán et. al., 2017).

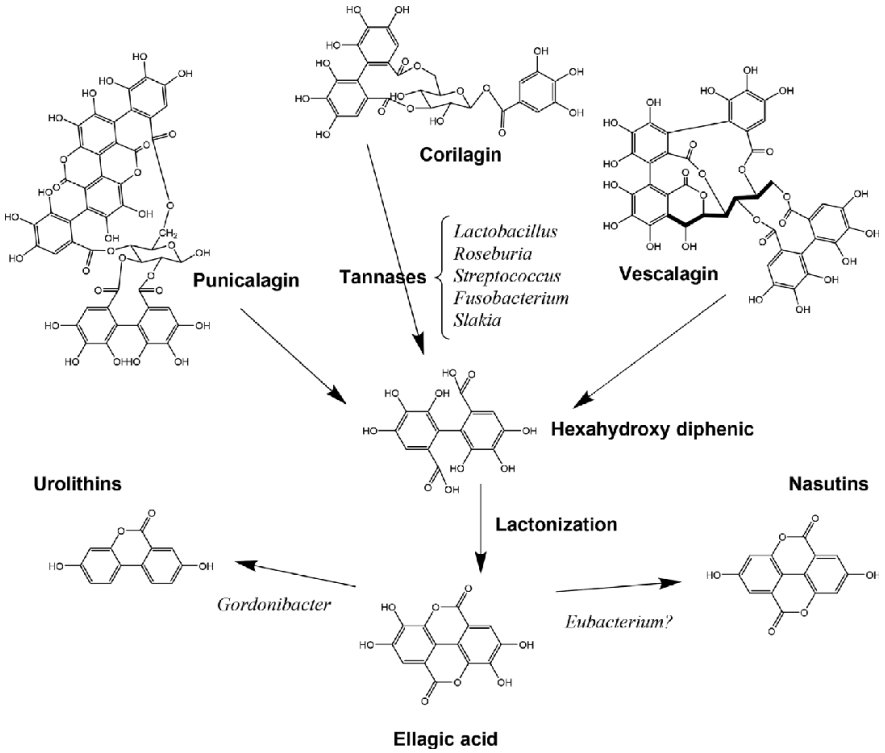

Figure 8. Transformation of ellagitannins into ellagic acid after hydrolysis and ellagic acid into nasutin and urolithin by intestinal microbiota (Tomás-Barberán et al., 2017).

Ellagic acid can also be transformed into pentahydroxy-urolithin (Uro-M5) by cleavage and decarboxylation of the lactone ring, from which successive dihydroxylations take place producing dihydroxy-urolithins (urolithin A, Uro-A and isourolithin A, IsoUro-A) and 3-hydroxy-urolithin (urolithin B, Uro-B), urolithins metabolites that have been detected in *in vivo* studies (**Figure 9**) **(**García-Villalba et al., 2019; Tomás-Barberán et al., 2017).

Studies with animals demonstrated that the formation of urolithins metabolites begins in the large intestine, suggesting that anaerobic bacteria are responsible for transforming ellagitannins and ellagic acid into urolithin (Sallam et al., 2021; Larrosa et al., 2010). In summary, ellagitannins and ellagic acid have low absorption and bioavailability. Therefore, their gut metabolites, urolithins, are possibly responsible for the beneficial health effects found after the consumption of food sources such as pomegranates, walnuts, and berries (Banc et al., 2023; Tomás-Barberán et al., 2017; Cardona et al., 2013). Urolithins are more lipophilic than ellagic acid and ellagitannins, a possible explanation for the higher rate of absorption of urolithin, reaching significant concentrations in blood and urine (Tomás-Barberán et al., 2017; García-Niño & Zazueta, 2015; García-Villalba et al., 2022).

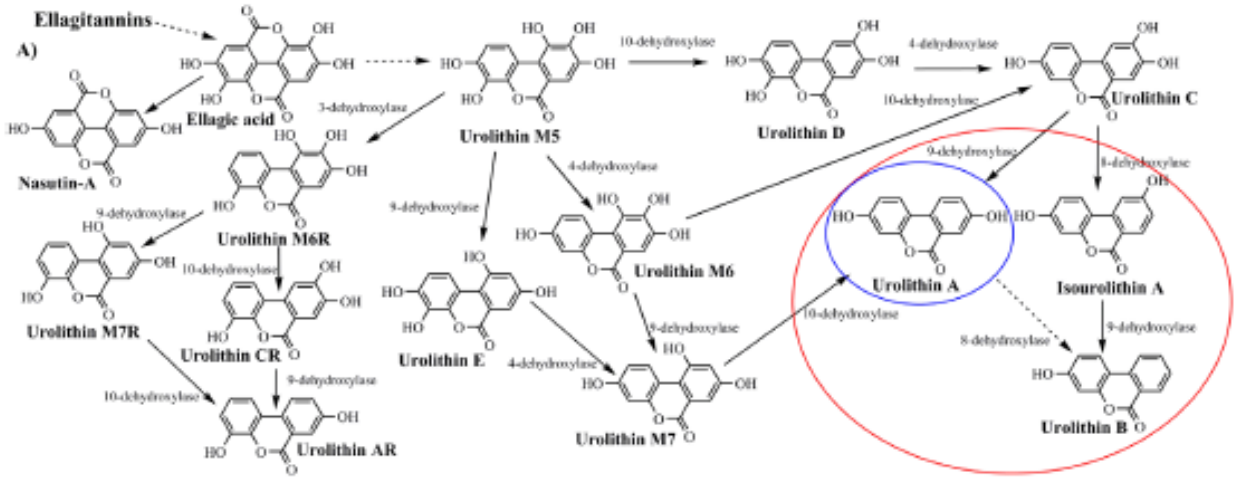

Figure 9. Urolithin formation from ellagic acid (Cortés-Martín, Selma, et al., 2020a). The circles highlight the final urolithins for each metabotype (red, UM-B; blue, UM-A).

5. Metabolism of urolithins

Urolithins are a subgroup of dibenzo [b, d] pyran-6-ones (also called 3,4-benzocoumarins or dibenzo-α-pyrones), which were first isolated in 1949 from animal tissues (beaver scent glands) and later confirmed in plants, microorganisms, and animal residues (González-Barrio et al., 2011; Lederer, 1949). After 31 years, Doyle & Griffiths (1980) demonstrated that for urolithins to be produced, the presence of bacteria was necessary because when carrying out experiments administering ellagic acid to wild-type and germ-free rats, Uro-A was produced by the former but not the latter. Corroborating with these results regarding the importance of the gut microbiota, the authors then incubated samples of human feces with ellagitannins or ellagic acid and also observed the production of urolithins (Cerdá et al., 2005). More recently, it was observed that in the administration of pomegranate, which is rich in ellagitannins and not free of ellagic acid, to both animals and humans, urolithins were detected in the plasma and urine (Cerdá et al., 2004; Cerdá et al., 2003).

The end products of the conversion of ellagic acid and ellagitannins by the intestinal microbiota are mostly Uro-A, IsoUro-A, and Uro-B. They are found in various biofluids (urine, feces, and blood) and organs (breast, colon, and prostate) in free or conjugated forms (glucuronide or sulfates). Urolithin aglycones undergo conjugation in the liver and the intestine through uridine 5'-diphospho (UDP)-UGT, which increases their solubility and subsequent excretion in the urine (González-Barrio et al., 2011).

Urolithins in biofluids probably persist for a few days after ingesting food rich in ellagitannins (Ludwig et al., 2015). Monohydroxylated Uros (Uro-B) was found in higher

proportion than tetrahydroxylated (Uro-M6, -D, -M7, -C) in 24-hour human urine samples after consumption of sources of ellagitannins, possibly due to its greater hydrophilicity (González-Sarrías et al., 2015). Additionally, tetrahydroxylated urolithins are intermediate metabolites, therefore, they are possibly further metabolized. Furthermore, due to the entire phase I and II processes, urolithins in plasma, tissues, and urine are found in higher levels as glucuronides than as aglycone (Tomás-Barberán et al., 2017). The concentration of urolithins ranges from 0.003 to 5.2 μM in the blood and up to 50 μM in the urine. Additionally, urolithins can reach tissues and organs such as the breast, colon, and prostate (García-Villalba et al., 2023; Ávila-Gálvez et al., 2019; Nuñez-Sánchez et al., 2014; González-Sarrías et al., 2010). Urolithin concentration in blood and urine can be affected by the source of ellagitannins, the amount that is consumed, the analytical methods used, and interindividual differences in the ADME process, mainly the gut microbiota (Villalba et al., 2019; Ludwig et al., 2015; Cerdá et al., 2005).

Cerdá et al. (2005) called urolithins the "missing link" to explain the health benefits of ellagitannins consumption (Espín et al., 2013; García-Villalba et al., 2022a; Savi et al., 2017; Tomás-Barberán et al., 2017; Espín et al., 2013). In this regard, the beneficial effects of ellagitannins consumption, such as anti-inflammatory, differ among individuals depending on the microbiota composition, which will determine the metabolism of ellagitannins to urolithins (García-Villalba et al., 2022a).

Some microbial strains have been correlated with urolithin levels. Among the bacterial species isolated from human feces, Butyrivibrio *spp*. and Lactobacilli present tannase activity capable of hydrolyzing ellagitannins. The species *Bifidobacterium pseudocatenulatum* INIA P815 and *Gordonibacter urolithinfaciens* can produce Uro-A and B from ellagic acid and ellagitannins, respectively (Gaya et al., 2018; Selma et al., 2014). The production of Uro-A in *in vitro* studies was proportional to the abundance of the genus *Gordonibacter* (Martinez-Blanch et al., 2017; Selma et al., 2014). Ellagitannins and ellagic acid can also be metabolized to urolithins and other metabolites by *Akkermansia muciniphila* and other bacterias (Henning et al., 2017). *Akkermansia muciniphila* was found in individuals that produced urolithins after consumption of pomegranate extract rich in ellagitannins and possibly can release ellagic acid from ellagitannins via enzymatic activity. The production of Uro-B and IsoUro-A was positively correlated with *Lactobacillus/Leuconostoc/Pediococcus* bacteria and also with strains of the *Eggerthellaceae* family (4A1, 4A2, 4A3, 4A4) (Romo-Vaquero et al., 2015). Uro-B and Uro-A were produced in the presence of ellagic acid and *Bifidobacterium pseudocatenulatum* INIA P815 (Gaya et al., 2018). Uro-M5, -M6, and -D production was

increased by Bacteroides, Uro-C by *Gordinobacter urolithinfaciens* and *G. pamelaeae* (Selma et al., 2017; Selma et al., 2014). However, a complete description of the microorganisms involved in the biotransformation of ellagic acid and ellagitannins into final urolithins is still needed (Selma et al., 2017).

The identification of bacterial strains that are involved in the production of urolithins is interesting to deepen the understanding of ellagic acid and ellagitannins metabolism and also to serve as an incentive for industries to formulate products with probiotic strains to increase the production of urolithins which would be even more relevant for individuals which are not able to produce them (UM-0) (Luca et al., 2020).

6. Stratification of the population into metabotypes

The term metabotype refers to the different metabolic responses of individuals to a nutritional or pharmacological intervention (Iglesias-Aguirre et al., 2021). This concept, introduced by Gavaghan et al. (2000), proposed a metabolomic approach to associate the metabolite profile that is excreted to the host phenotype and genotype (Selma et al., 2017). Years later, the metabotype concept expanded to a transgenomic approach following the proposal by Bolca et al. (2013) in an attempt to connect the microbiome to the phenotype.

In the context of health and phenolic compounds, metabotype refers to metabolic phenotypes associated with the production of specific metabolites of the parent compound and the associated gut microbiota in terms of composition and activity (Iglesias-Aguirre et al., 2022; Espín et al., 2017). It consists of subpopulations of individuals that have a distinct and well-characterized metabolization of some classes of polyphenols, determined by the composition of the gut microbiota (González-Sarrías et al., 2017a; Narduzzi et al., 2022). Additionally, metabotypes have been associated with genetic polymorphisms, such as the polymorphism in cytochrome P450 1A2 (Landberg et al., 2019; Palatini et al., 2009).

External factors have less impact on metabotypes, being associated with a qualitative criterion (producers and non-producers). The classification into metabotypes can be evaluated in different body fluids (plasma, urine, feces, tissues) after ingesting specific plant foods or bioactive compounds (Cortés-Martín et al., 2020b; Landberg et al., 2019).

Several works have already been carried out demonstrating the metabolism and gut metabolites of some phenolic compounds generated after the ADME process, such as stilbenes (Jarosova et al., 2019), apigenin (Borges et al., 2022), proanthocyanidins (Cortés-Martín et al.,

2019), isoflavones (Iglesias-Aguirre et al., 2021) ellagitannins (Tomás-Barberán et al., 2014) among others (Cortés-Martín et al., 2019). However, a gut metabolite to qualify as a marker of a metabotype must be a distinct and exclusive metabolite of the original phenolic compound present in the diet rather than a metabolite produced through microbial metabolism shared by other phenolic compounds (Cortés-Martín, Selma, et al., 2020a).

For the intake of isoflavones, the classification of subjects into metabotypes refers to the production or not of equol and/or *O*-desmethylangolesin (ODMA) as a result of daidzein metabolism (Mayo et al., 2019; Frankenfeld, 2017). These two metabolites are separately produced by distinct bacteria (**Figure 10**) (Frankenfeld, 2011). Equol producers correspond to about 30% of the Caucasian population and 50-60% of the Asian population, partly explaining the discrepancies in the effects of daidzein in human studies (Iglesias-Aguirre et al., 2021). The difference between these two populations suggests that equol production may be linked to genetic background, microbiota composition, and dietary habits since the Asian population is more exposed to foods rich in isoflavones (Iglesias-Aguirre et al., 2021).

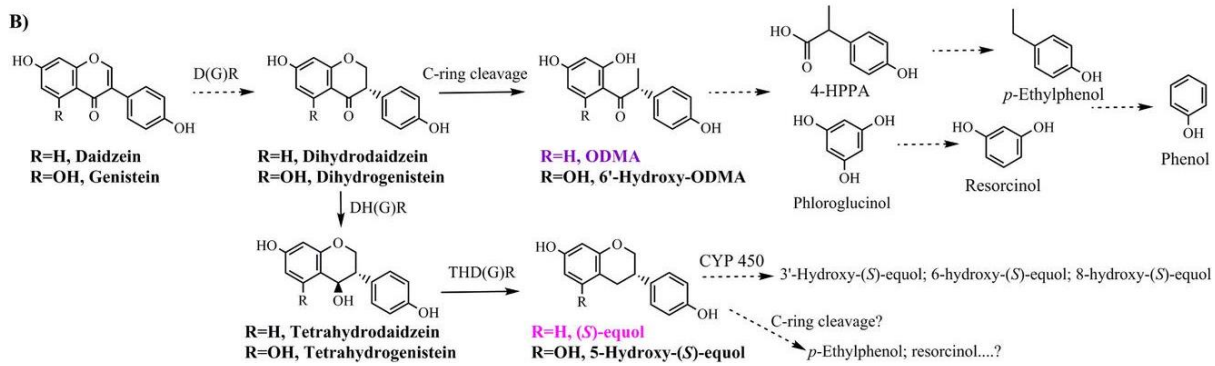

Figure 10. Production of equol and ODMA from daidzein and genistein (Cortés-Martín et al., 2020a). D(G)R, daidzein/genistein reductase; DH(G)R, dihydrodaidzein/genistein reductase; THD(G)R, tetrahydrodaizein/genistein reductase; CYP450, mammalian cytochrome P450; 4-HPPA, 4-hydroxyphenyl propionic acid.

The influence of race, sex, ethnicity, body composition, or age on the classification of metabotypes by equol or ODMA producers has not yet been described (Li et al., 2020; Frankenfeld, 2017; Frankenfeld et al., 2014). In the Caucasian population, ODMA producers after consumption of soy-rich products corresponded to 80-90% (Frankenfeld et al., 2014). It is currently unclear the proportion of metabotypes stratified by equol and ODMA producers from

isoflavones since ODMA can generate minor metabolites that are similar to the microbial catabolism of other phenolic compounds, thus possibly altering the distribution of the ODMA metabotype in the studied population (Cortés-Martín, Selma, et al., 2020a). S-equol can be hydroxylated by CYP; however, no minor metabolite has been identified (Cortés-Martín, Selma, et al., 2020a)

More recently, two new stratifications of metabotypes were proposed based on metabolites of citric flavones and resveratrol (Iglesias-Aguirre et al., 2022; Nishioka et al., 2021). Nishioka et al., 2021 evaluated the consumption of two juice sources of hesperidin (orange "Pera" and orange "Moro") in twenty-seven women divided into obese and non-obese. With the result of the study, they divided the group into Profile A and Profile B, where the latter had a greater capacity to transform hesperetin sulphate into hesperetin sulfo-*O*-glucuronide. They did not find a direct relationship between the intestinal microbiota profile and BMI, and for the first time, suggested that the profile of flavanone metabolites would be associated with polymorphism in phase II enzymes, mainly UDP-UGT. In this case, the separation was not qualitative; since both groups excreted hesperetin sulfo-*O*-glucuronide, it was a quantitative extraction.

Iglesias-Aguirre et al. (2022) evaluated the metabolism of resveratrol in a large group of individuals (n = 195) who consumed capsules containing 150mg of resveratrol extracted from the *Polygonum cuspidatum* plant for seven days, and the urinary excretion of the metabolites was evaluated. They were stratified into lanularin producers and non-producers. Only the producers were able to excrete the metabolite 3,4'-dihydroxy-trans-stilbene and conjugates and 4-hydroxydibenzyl and conjugates. According to the author, the remarkable capacity of dehydroxylation (lanularin producers) of resveratrol is intrinsic to the individual, probably related to the intestinal microbiota, without being directly affected by external factors, which would fit the concept of metabotype. The division found that 74% of the volunteers, after consuming resveratrol, produced this metabolite, while 26% did not have these dehydroxylase activities. Within this 26%, most were women, and it was not possible to identify a relationship with age or BMI. As it is the first work to describe the detailed process of resveratrol metabolism, more work needs to be done to evaluate the real effect of these metabolites on health.

6.1 Urolithin metabotypes

In the case of ellagitannins and ellagic acid there are three metabotypes related to urolithins production: metabotype A (UM-A), producers of urolithin A, metabotype B (UM-B) producers of Uro-A and Uro-B, and/or IsoUro-A and metabotype 0 (UM-0) (non-producers of Uro-A and Uro-B, and/or IsoUro-A). In metabotype 0, only the pentahydroxy-urolithin precursor can be detected (García-Villalba et al., 2022; Cortés-Martín et al., 2020a; Tomás-Barberán et al., 2014). More recently, new metabolites were identified in human feces, namely urolithin M6R (4,8,9,10-tetrahydroxurolithin), M7R (4,8,9-trihydroxyurolithin), and urolithin AR (4,8-dihydroxyurolithin), which are common to individuals UM-A and UM-B, and bacterial 3-dehydroxylase activity is required for their production. However, the study was conducted with a limited number of participants, and further studies are necessary to demonstrate these new metabolites' fecal and urinary excretion (García-Villalba et al., 2019).

The frequency of urolithin metabotypes within the population in Europe, Asia, and, more recently, the Brazilian population, ranged from 5-30% UM-0, 29-81% UM-A and 8-56% UM-B (**Table 1**).

Table 1. Distribution of urolithin metabotypes in humans according to age, BMI, health

Metabotype distribution (%)			Age (years)	Sex		BMI (kg/m^2)	Health status	Nationality	Biofluid	Source	Time of consumption	Reference
A	B	0		Male	Female							
50	41	9	31-63	23	18	31.0±2.9	MetS	Spain	Urine (24 h)	30 g of mixed nuts (81.4 mg of EA/day)	12 weeks	Tulipani et al., (2012)
80	15	5	25-30	8	12	18.5-24.99	Healthy	Spain	Urine (92 h)	200 g of fresh and strawberry puree (31 mg of total EA/day)	4 weeks	Truchado et al., (2012)
25	50	25	50-65 (53.0±6.5)	10	10	26-39 (31.8±4.4)	MetS	Finland	Urine	300 g of fresh berries - 100 g of strawberry purée, 100 g of frozen raspberries, and 100 g of frozen cloudberries (789 mg of ET/day)	8 weeks	Puupponen -Pimiä et al., (2013)
42	42	16	52-89	14	12	28.9±3.9	Colorectal cancer	Spain	Urine	450 mg of PE (110 mg/day)	5 to 35 days	Nuñez-Sánchez et al., (2014)
80	10	10	18-23	11	9	20.5±2.1	Healthy	Spain	Urine (24h)	PE (220 mg of total EA/day)	3 days	Tomás-Barberán et al., (2014)

Table 1. Distribution of urolithin metabotypes in humans according to age, BMI, health (*cont*.).

Metabotype distribution (%)			Age (years)	Sex		BMI (kg/m^2)	Health status	Nationality	Biofluid	Source	Time of consumption	Reference
A	B	0		Male	Female							
79	14	7	11-14	65	74	21.5±0.9	Healthy	Spain	Urine (24h)	25g of Walnuts (102.5 mg of free EA /day) or 250 mL of PJ (75.8 mg of free EA /day)	3 days	Cortés-Martín et al., (2018)
77	14	10	15-19	63	63	23.8±2.7	Healthy	Spain	Urine (24h)	25g of Walnuts (102.5 mg of free EA/day) or 250 mL of PJ (75.8 mg of free EA /day)	3 days	Cortés-Martín et al., (2018)
52	41	7	20-72	61	158	27.4±3.3	Healthy	Spain	Urine (24h)	30g of Walnuts (123 mg of free EA /day) or 450 mg of PE (218.4 mg of free EA /day)	3 days	Cortés-Martín et al., (2018)
30	40	30	25-60	6	4	NM	Healthy	NM	Urine	400 mL of yellow, black, dark, white, green and oolong (3mg of EA)	2 days	Yang & Tomás-Barberán, (2019)

Table 1. Distribution of urolithin metabotypes in humans according to age, BMI, health (*cont*.).

Metabotype distribution (%)			Age (years)	Sex		BMI (kg/m^2)	Health status	Nationality	Biofluid	Source	Time of consumption	Reference
A	B	0		Male	Female							
54	29	17	20-45 (27.5)	18	17	18.5-25 (23±2) and >27 (34±6)	Healthy and OW	Brazil	Urine (48h)	20 g of JPSP (1,493 mg of total PC)	Acute	Inada et al., (2019)
58	34	8	19−55 (36.9±9.0)	23	27	18.4−34.3 (23.7±3.2)	Healthy	Spain	Urine (24h)	30 g of unpeeled walnuts (162.8 mg of free EA /day)	3 days	Romo-Vaquero et al., 2019
79	14	7	11-14	65	74	21.5±0.9	Healthy	Spain	Urine (24h)	25g of Walnuts (102.5 mg of free EA /day) or 250 mL of PJ (75.8 mg of free EA /day)	3 days	Cortés-Martín et al., (2018)
53	33	14	40−65 (46.2±6.3)	32	17	27.1−43.0 (30.4 ± 3.4)	Healthy and OW	Spain	Urine (24h)	450 mg of PE (45.4 mg of free EA /day)	3 days	Romo-Vaquero et al., (2019)
53	37	10	19−72 (43.3±12.8)	51	99	18.0−39.9 (27.5±4.7)	Healthy and OW	Spain	Urine (24h)	1,350 mg of PE (136.2 mg of free EA /day)	3 days	Romo-Vaquero et al., (2019)

Table 1. Distribution of urolithin metabotypes in humans according to age, BMI, health (*cont*.).

Metabotype distribution (%)			Age (years)	Sex		BMI (kg/m^2)	Health status	Nationality	Biofluid	Source	Time of consumption	Reference
A	B	0		Male	Female							
44	56	0	27	NM	27	19.5–33.3 (25.8±3.5)	Healthy	Spain	Breast milk	30 g of Peeled walnuts (123 mg of free EA /day)	3 days	Cortés-Martín et al., (2020b)
54	23	23	>18 52.9±9.4	NM	26	27.3±5.3	hysterectomy patients	Slovakia	Blood	300 mg Proprietary oak wood extract (Robuvit®)	8 weeks	Volpp et al., (2020)
63	13	25	47-73	40	NM	31	Prostate cancer	USA	Urine (24h)	10 or 20g of Black raspberry nectar or confection (25 to 50 mg ETs)	3 weeks	Roberts et al., (2020)
40	40	20	35±2	14	11	28.0±1.0	prediabetes and insulin-resistance	USA	Urine (24h)	250 g of red raspberry drink (388.4 ± 3.3 of PC)	Acute	Zhang et al., (2020)
50	40	10	31±3	3	7	22.0±1.0	Healthy	USA	Urine (24h)	250 g of red raspberry drink (388.4 ± 3.3 of PC)	Acute	X. Zhang et al., (2020)

Table 1. Distribution of urolithin metabotypes in humans according to age, BMI, health (*cont*.).

Metabotype distribution (%)			Age (years)	Sex		BMI (kg/m^2)	Health status	Nationality	Biofluid	Source	Time of consumption	Reference
A	B	0		Male	Female							
37	44	19	33±7	4	12	23.0±3.0	Healthy	Brazil	Urine (24h)	300 mL of non-clarified cagaita juice (0.87 ± 0.04g of GAE	Acute	Araujo et al., (2021)
29	42	29	45±11	NM	12	37±8	MetS	Brazil	Urine (24h)	300 mL of non-clarified cagaita juice (0.87 ± 0.04g of GAE	Acute	Araujo et al., (2021)
54	32	14	21-30 (24.0±2.0)	15	20	16.9-24.8 (20.5±1.9)	Healthy	China	Feces	PE (70% of EA)	3 days	Xian et al., (2021)
65	25	10	70–87 (77 volunteers)	NM	NM	23-30	Adults with "leaky gut"	Italy	Urine (24h)	Polyphenol rich diet (pomegranate juice, berries pureé, green tea, blueberry) 0.5-6.7 mg/100 g of FW	8 weeks	Meroño et al., (2022)

BPH: Benign prostatic Hyperplasia; EA: ellagic acid; ET: ellagitannins; FW: fresh weight; GAE: gallic acid equivalent; JPSP: jaboticaba peel and seed powder; M: men; MetS: metabolic syndrome; NM: not mentioned by the authors; OW/OB: overweight and obese; PC: Prostate cancer PE: pomegranate extract; PJ: Pomegranate juice; PC: phenolic compounds; USA: United States of America.

The first studies to evaluate the distribution of urolithins metabotype took place in Europe, mainly in Spain, by analyzing the 24 h urinary excretion of urolithins after the consumption of different ellagitannins sources, such as nuts, pomegranate, strawberry, blackberry, and tea. More recently, native Brazilian fruits such as jaboticaba and cagaita were also used to assess the urolithins metabotype distribution, especially in the Brazilian population (**Table 1**).

Overall, compiling the results presented above, it can be concluded that almost all individuals were capable of synthesizing urolithins, representing more than 80% of the subjects participating in the studies.

In healthy adults, age was an important factor to classified individuals into metabotypes: In this regard, in a cohort of 839 subjects and age between 5-90 years, it was observed that the proportion of UM-B individuals increased from 15% to up to 45% as a function of aging, in contrast to the reduction of UM-A individuals from 85% to 55%, in particular in the range of 20-40 years old (Iglesias-Aguirre et al., 2021; Cortés-Martín et al., 2018). Age seems to be a contributing factor for the stratification of different urolithin metabotypes for subjects with metabolic and inflammatory diseases.

The investigations were carried out in healthy adults and those with metabolic syndrome (MetS). When they were divided by age, young individuals (≤ 40 years old) (n = 9) were distributed into 60% UM-A, 30% UM-B and 10% UM-0, whereas the distribution of adults older than 40 years old (n = 3) was 47% UM-A, 38% UM-B and 15% UM-0 (Cortés-Martín et al., 2018). Some authors analyzed the metabotype of healthy overweight young adults and found it to be of 53% UM-A, 33% UM-B, and 14% UM-0 (n = 3) (Cortés-Martín et al., 2018). Some studies presented on **Table 1** were carried out with individuals with overweight with diseases associated with dysbiosis, such as "leaky gut" and insulin resistance. Most of them were adults with overweight older than 40 years old (n = 9) were stratified as UM-A 47%, UM-B 35% and UM-0 18% (Meroño et al., 2022; Araujo et al., 2021; Zhang et al. 2020; Volpp et al., 2020; Roberts et al., 2020; Selma et al., 2018; Nuñez-Sánchez et al., 2014; Puupponen-Pimiä et al., 2013; Tulipani et al., 2012)

Most studies demonstrated that healthy individuals were most frequently UM-A and UM-B individuals had some associated pathology or dysbiosis. However, this association was not confirmed in the large cohort carried out by Cortés-Martín et al., (2018). Likewise, factors such as sex, dietary pattern or ethnicity were not confirmed to be associated with urolithin metabotypes (Cortés-Martín et al., 2020b; Cortés-Martín et al., 2019).

7. Importance of subdividing the population into metabotypes

Dietary intervention studies with humans, mainly epidemiological studies addressing cardiovascular diseases, have shown discrepant and possibly underestimated responses between individuals (Manach et al., 2017; Zeevi et al., 2015). Therefore, caution is needed while interpreting these results so that one does not underestimate the magnitude of the effect of phenolic compounds consumption on specific markers of cardiovascular health (Morand & Tomás-Barberán, 2019; Milenkovic et al., 2017).

An example to be highlighted refers to the meta-analysis that evaluated the effect of consumption of food sources of ellagitannins on cardiovascular health, which failed to find a significant effect on the lipoprotein profile since most studies included in this review did not use the stratification of individuals into urolithin metabolites (UM- A, UM-B, and UM-0) (García-Conesa et al., 2018).

These discrepancies found in many studies regarding the health effects of phenolic compounds on humans may be a consequence of interindividual variations in the entire ADME process, particularly the role of the colonic intestinal microbiota (Manach et al., 2017). The high dispersion of data among subjects in well-controlled studies is one aspect that favors huge interindividual variations in the ADME process (Morand & Tomás-Barberán, 2019).

The use of dietary questionnaires, food composition tables, and software to evaluate the consumption of bioactive compounds may not be reliable in a clinical trial by not being faithful to what is excreted by individuals (González-Sarrías et al., 2017b; Cassidy & Minihane, 2017). With this, evaluating this exposure in biofluids such as urine can better reflect the exposure to phenolic compounds based on the profile of metabolites present and then associate with beneficial health effects.

In this regard, several studies indicate the need to group individuals into metabotypes, which could help explain, even partially, the interindividual variability observed after the consumption of food rich in phenolic compounds and possibly decrease confounding factors that may tamper the response to the intervention (García-Mantrana et al., 2019; Morand & Tomás-Barberán, 2019).

The characterization of the microbial metagenomic profile faces a challenge due to the lack of a database. The Cooperation in Science and Technology-Positive group (COST-Positive) (Landberg et al., 2019; Morand et al., 2020), a large European group created to build a scientific network to propose strategies to understand the determinants of interindividual

variation in phenolic compounds health effects. COST-Positive is formed by three subgroups: G1 group is responsible for addressing the factors that can affect the interindividual variability in the ADME process; G2 is responsible for compiling the main determinants related to the biotransformation of the human intestinal microbiota in specific bioactive metabolites; and the G3 group is responsible for developing the framework for the metabolomics approach to be used to assess bioactive metabolites in each individual and their interindividual variability. The COST-Positive initiative was created to work with industries and regulatory bodies to translate scientific literature into product innovation and dietary recommendations (Landberg et al., 2019; Morand et al., 2020).

It is mandatory that interindividual variability in the ADME of phenolic compounds continues to be investigated in depth and that it is considered in the study design to help achieve a better stratification of subjects and interpretation of results. By doing so, it is anticipated that dietary guidelines for phenolic compounds consumption may arise and that the food industry will be encouraged to innovate in products that deliver more health benefits to consumers, and possibly consumers will be convinced to adopt the recommended eating habits (Morand et al., 2020; Landberg et al., 2019).

Instead of providing the population with guidance on fruit and vegetable consumption in general, it would be particularly relevant to offer guidance on phenolic compounds-rich diets tailored to specific individuals stratified according to metabotype so that everyone is adequately exposed to the protective components of foods from the plant (Morand & Tomás-Barberán, 2019). This strategy would lead to the prevention and management of some diseases based on "personalized nutrition", leading to an ideal strategy that would bring health benefits to each individual (Palmnäs et al., 2020; Cortés-Martín et al., 2020a; Hillesheim & Brennan, 2020; Kolodziejczyk et al., 2019; Espín et al., 2017). By using personalized and targeted nutritional recommendations, consumers tend to improve their behavior (Narduzzi et al., 2022; Hillesheim & Brennan, 2020).

8. Importance to assess metabotype change and the degree of excretion

The production of urolithins metabolites is the probable link to the beneficial health effects of ellagitannins consumption, such as anti-inflammatory, antioxidant, and neuroprotective effects (Banc et al., 2023; Tomás-Barberán et al., 2017). Among the urolithins metabotypes found, UM-A appears to have a protective intestinal microbiota profile, and UM-

B is potentially more prone to dysbiosis and cardiovascular risk (Iglesias-Aguirre et al., 2021; Cortés-Martín et al., 2020a; Romo-Vaquero et al., 2019).

González-Sarrías et al. (2017b) evaluated the effect of pomegranate extract consumption on cardiovascular markers. Two doses of pomegranate extract (160 mg phenolics/day versus 640 mg phenolics/day) were administered for 3 weeks with a 6-week interval between doses (3 weeks with no intervention and 3 weeks with placebo) in adults with overweight/obesity (BMI>27 kg/m^2) with an average age of 40 years old. They reported improvement in cardiovascular risk biomarkers (total cholesterol, LDL-cholesterol, HDL-non-cholesterol, apolipoprotein B, and oxidized LDL-cholesterol) in UM-B subjects after consumption of the high-dose and no significant effect was found in UM-A subjects. These results were correlated with the conjugated urolithins excreted in the urine, and this relationship could be casual and not causal (García-Villalba et al., 2022). With the exposed data, it is possible to arrive at three relevant results about cardiovascular risk: to observe the positive effect of the consumption of food sources of ellagitannins, stratification into metabotypes is necessary; higher doses of the same matrix are capable of bringing more pronounced results for reduction of cardiovascular biomarkers, and UM-B individuals may benefit more from consuming these foods.

Another relevant piece of information derived from the studies of metabotypes of urolithins was the possibility of metabotype conversion. Three individuals initially categorized as UM-0, *i.e.*, unable to produce urolithins, became urolithin producers or responders. Two individuals were converted to the metabotype UM-A and one individual to the UM-B after ingesting the highest dose of pomegranate extract (640 mg phenolics/day) in the study. These results suggest that these individuals excreted urolithins at very low levels, to the point of not being detected by the analytical method (Narduzzi et al., 2022; González-Sarrías et al., 2017). Therefore, one can speculate that UM-0 individuals would benefit from high doses or long-term exposure to food rich in ellagitannins and ellagic acid.

Other clinical studies of chronic consumption were performed in humans (doses between 25-789 mg EA/ET) (**Table 2**). Chronic exposure of ellagitannins to human health was also evaluated. Of the articles cited below that also evaluated the chronic effect of ellagitannins exposure, three did not describe a baseline value that would allow comparison. One of the works that presented values that allow comparison, Roberts et al. (2020), highlights a significant increase in the excretion of urolithins after 4 weeks of intervention with both doses and forms of administration of ellagitannins. Kresty et al. (2016) analyzed excretion over a 14-week

interval and did not identify a significant difference between the two points, even using the same food source as Roberts et al. (2020). Such differences may be due to the way they were administered (dehydrated powder, lyophilized powder, jelly, extract), as well as to the interindividual variability that can arise from the intestinal microbiota, genetic background, and diet (Sallam et al., 2021; Morand & Tomás-Barberán, 2019; Cerdá et al., 2005). Evaluating the modification of the individual metabotype, especially UM-0 (non-producers), to urolithin producers and the level of excretion of metabolites after chronic consumption is important to future studies associating these metabolites with health effects.

Table 2. Clinical studies evaluating the urolithin urinary concentration after chronic consumption of ellagitannin sources[1].

Number of volunteers	**Dose administered/Source**	**Intervention time (weeks)**	**Total urolithins quantification**	**Reference**
22 MetS patients	30 g/day of mixed nuts (37.8 mg of EA equiv.)	12	92.4 µmol[2]	Tulipani et al., (2012)
20 healthy Volunteers	1 g/day of PE (70 mg of punicalagin and 68 mg of EA)	4	76 µmol	Li et al., (2015)
20 Barrett´s esophagus Patients	Black raspberries powder (32 g for WM and 45 g for M)	12 and 26	3.52 µmol (week 12) 4.68 µmol (week 26)	Kresty et al., (2016)
40 cancer prostate patients	Pectin confection (C) or nectar (N) of black Raspberry (10 or 20 g day−1) (25 or 50 mg ETs + EA)	3	Baseline: 0.03 µmol N10: 1.60 µmol N20: 4.44 µmol C10: 3.34 µmol C20: 5.18 µmol	Roberts et al., (2020)

[1]Table adapted from Tomás-Barberán et al. (2017) and Garcia-Villalba et al. (2022). [2]Concentration was determined after enzymatic treatment with acetic acid. C: Pectin Confection; EA: ellagic acid; ET: ellagitannins; equiv.: equivalents; M: men; MetS: metabolic syndrome; PE: pomegranate extract; WM: women.

The potential of jaboticaba peel and seed powder consumption to modify urolithin excretion and metabotype: a clinical study controlled by placebo.

Manuscript to be submitted to the *Journal of Functional Foods*

1. Introduction

The various beneficial health effects associated with the consumption of fruits and vegetables are due, in part, to the presence of phenolic compounds. These compounds have been associated with preventing non-communicable diseases, such as cardiovascular diseases, diabetes, and cancer, in *in vitro* and *in vivo* models (Ubago-Guisado et al., 2021; Godos et al., 2019). However, clinical trials regarding the health effects of polyphenols-rich foods have shown inconclusive results, possibly due to interindividual variability in the gut microbiota composition (Landberg et al., 2019; Hamauzu, 2018).

During humans' metabolization of phenolic compounds, several metabolites produced by the gut microbiota presented different biological activities among individuals (Morand & Tomás-Barberán, 2019; Bouayed et al., 2011). These metabolites have already been identified in different biofluids, including plasma, feces, and urine (Landberg et al., 2019). Studies have classified the population into metabotypes, that is, grouping individuals based on the similarities of metabolic phenotypes, which may be the result of the interaction between lifestyle, genetics, environment, and gut microbiota composition (Singh et al., 2022; Palmnäs et al., 2020; García-Villalba et al., 2019; Espín et al., 2017; Brennan, 2017). This stratification seems necessary to understand the health effects of certain phenolic-rich foods (González-Sarrías et al., 2017a).

Urolithins are ellagitannins and ellagic acid metabolites produced by the intestinal microbiota. The classification of individuals according to their metabotype refers to metabotype A (UM-A; producers of Uro-A), metabotype B (UM-B; producers of Uro-A, IsoUro-A, and Uro-B) and metabotype 0 (UM-0; non-urolithin producers) (Inada et al., 2019; Tomás-Barberán et al., 2014). Several factors seem to affect the profile and the amount of urolithin production/excretion, of which the gut microbiota stands out (García-Villalba et al., 2019).

Regarding the production of urolithins and their bioactivity, it has already been described that individuals with UM-B have a higher risk of cardiovascular disease compared to UM-A, and overweight and obese individuals were most frequently classified with UM-B (Selma et al., 2018; González-Sarrías et al., 2017a). Changes in metabotype, including the conversion of UM-0 individuals into UM-A or B, have already been reported after the chronic consumption of berries (González-Sarrías et al., 2017a; Puupponen-Pimiä et al., 2013).

The most frequently studied foods to assess the metabolism of ellagitannins are walnuts, pomegranate, green tea, and blueberry, and almost all of the studies have been carried

out with the European population (Yang & Tomás-Barberán, 2019). The first study on ellagitannins metabolism in humans to be conducted with Brazilian subjects was carried out by our research group with *M. jaboticaba* (Inada et al., 2019), a Brazilian berry in which ellagitannins are mainly concentrated in the fruit peel and seed (Inada et al., 2020; Quatrin et al., 2019; Pereira et al., 2017).

The present study aims to evaluate whether the 3 weeks consumption of jaboticaba peel and seed power (JPSP) by normoweight and overweight/obese adults Brazilian volunteers influences the metabotype and the amount of urolithins excreted in the urine.

2. Material and Methods

2.1 Standards and chemicals

Urolithin-A, ellagic acid, gallic acid, and vescalagin, cyanidin-3-*O*-glucoside and delphinidin-3-*O*-glucoside were purchased from Sigma-Aldrich (St. Louis, Mo, USA). Solvents and water (Milli-Q system, Millipore, Bedford, MA, USA) were HPLC grade.

2.2 Production and stability of jaboticaba peel and seed powder (JPSP) capsule

Jaboticaba fruits (*Myrciaria jaboticaba*, cv. Sabará) were purchased from Rio de Janeiro's agricultural trading center, washed, and sanitized in sodium hypochlorite 100 ppm solution for 15 min. Fruits were used to produce a steam-extracted juice, according to Martins et al. (2021), and peel and seeds were obtained as a co-product, which was oven-dried in a forced air circulation oven (Tecnal®, Piracicaba, Brazil) at 75 °C for 22 h (Inada et al., 2020), and then milled to obtain JPSP. The JPSP microbiological quality was evaluated by analyzing *E. coli*, *Salmonella spp*., molds, and yeasts and was considered adequate according to the current Brazilian legislation (Brasil, 2001).

JPSP was encapsulated in dark purple opaque gelatin capsules (each containing 0.8 g) following hygienic-sanitary quality and safety standards. To evaluate the stability of phenolic compounds, JPSP capsules were stored either at room temperature (~25 °C) or in a domestic fridge (~5 °C) in closed flasks for 57 days. Phenolic compounds were analyzed at the beginning and at the end of this period, following the procedure detailed below.

The extraction step followed the methodology described by Inada et al. (2015) for anthocyanins analysis, in triplicate. Briefly, JPSP (150 mg) was extracted with 40 mL of methanol:water (50:50, *v*/*v*) using an Ultraturrax extractor T18 BASIC (IKA®, Staufen, Germany) at 14,000 rpm for 1 min. After centrifugation (1,700 × *g*, 10 min, 20 °C), the residue was re-extracted once with 40 mL of methanol:water (50:50, *v*/*v*), centrifuged again, and supernatants were combined. The extraction procedure was repeated with acetone:water:acetic acid (70:29.5:0.5, *v*/*v*/*v*) as many times as necessary to obtain a colorless supernatant. The final extract was filtered with a 0.45 μm PTFE filter (Analítica®, São Paulo, Brazil) before analysis by high-performance liquid chromatography (HPLC).

For the analysis of soluble phenolic compounds, the extraction followed the methodology described by García-Villalba et al. (2015), in triplicate. Briefly, JPSP (150 mg) was extracted with 10 mL of 70% methanol for 1 min and centrifuged (4,696 × *g*, 10 min, 20 °C). The supernatant was collected and filtered with a 0.22 μm PVDF filter (Millipore®, Barueri, Brazil). Total extraction of phenolic compounds, which comprises soluble and insoluble compounds, was performed according to García-Villalba et al. (2015), in triplicate, by adding 5 mL of 4 M HCl to 50 mg of JPSP, in triplicate, vortexing for 1 min, and incubating at 90 °C for 4 h. After incubation, samples were cooled to room temperature, and pH was adjusted to 2.5 with 10 M NaOH solution. Samples were centrifuged (2,500 × *g*, 10 min, 10 °C), the supernatant was collected, and the volume was adjusted to 10 mL with water and filtered with a 0.22 μm PVDF filter. The pellet was reconstituted with 10 mL of methanol:DMSO (50:50, *v*/*v*), vortexed for 2 min, and kept in an ultrasonic bath for 15 min. After centrifugation (2,500 × *g*, 10 min, 10 °C), the supernatant was collected and filtered with a 0.22 μm PVDF filter.

The liquid chromatography system (Shimadzu®, Kyoto, Japan) included two parallel LC-20AD pumps, an SIL-20AHT automatic injector, a CBM-20A system controller, a DGU-20A5 degasser, and an SPD-M20A diode-array detector (DAD). Chromatographic separation of anthocyanins was performed according to Inada et al. (2015), with slight modifications. A reverse phase column (C18, 5 μm, 150 mm × 4.6 mm, Phenomenex) was used. The mobile phase consisted of a gradient of 1% formic acid and 2% acetonitrile in water (eluent A) and 1% formic acid and 2% acetonitrile in methanol (eluent B), with a flow rate of 1.0 mL/min. Prior to the injection, the column was equilibrated with 23% B. After injection, the solvent composition was kept constant until 1 min, increased to 29% B in 2 min, to 33% B in 4 min, to 48% B in 6 min, to 8% B in 8 min, and to 95% B in 10 min. Then, it decreased to 23% B in 11

min. Between injections, 10 min intervals were used to re-equilibrate the column with 23% B. Anthocyanins were monitored by DAD at 530 nm.

Chromatographic separation of ellagic acid, gallic acid, and vescalagin was achieved using a reverse phase column (C18, 5 μm, 250 mm × 4.6 mm, Kromasil®). The mobile phase consisted of a gradient of 0.3% formic acid and 1% acetonitrile in water (eluent A) and 1% acetonitrile in methanol (eluent B), with a 1.0 mL/min flow rate. Prior to the injection, the column was equilibrated with 18.2% B. After injection, the solvent composition was increased to 20.2% B in 1 min, to 43.4% B in 18 min, to 85.9% in 23 min, and kept constant until 30 min. Between injections, 10 min intervals were used to re-equilibrate the column with 18.2% B. Ellagitannins and ellagic acid derivatives were monitored by DAD at 280 and 360 nm, respectively.

All compounds were identified by comparison with their respective commercial standards' retention time and absorption spectrum. Quantification was performed by external calibration. Data was acquired by LC solution software (Shimadzu Corporation®, version 1.25, 2009).

2.3 Clinical study

The clinical trial was conducted according to the ethical principles for medical research involving human subjects outlined in the Declaration of Helsinki, being approved by the research ethics committee of Clementino Fraga Filho Hospital from the Federal University of Rio de Janeiro, Brazil (number: 24043219.5.0000.5257). The volunteers signed an informed consent form (**Annex 1**).

Men and women were recruited by direct approach considering the following inclusion criteria: body mass index (BMI) between 18.5 kg/m^2 and 40.0 kg/m^2 and age between 19 and 59 years old. Non-eligibility criteria included pregnant or lactating women, volunteers who had been on weight loss diets in the last three months, who had difficulty ingesting capsules or tablets, who reported food allergies and chronic diseases (especially liver diseases), smokers, alcoholics, and those who reported routine or recent (last three months) use of medicines, antibiotics, anti-inflammatories, antifungals, probiotics, or nutritional supplements.

From the 64 volunteers that were recruited, 4 were not eligible due to antibiotics use during the study, 1 was not eligible due to difficulties in consuming the capsules, and 59 concluded the study. The BMI of each volunteer was calculated from their weight (measured

on a scale) and height (self-informed), and their percentage of body fat was measured by bioimpedance using a BC-601 scale (Tanita®, Tokyo, Japan). Twenty-four volunteers (41%) were classified as normoweight (NW) (BMI 22.8 ± 1.4 kg/ m^2), being 15 women and 9 men, with a mean age of 32 years. Thirty-five volunteers (59%) were classified as overweight/obese (OW/OB) (BMI 28.9 ± 2.8 kg/ m^2), being 16 women and 19 men, with a mean age of 34 years. Thirty volunteers (51%) had adequate % body fat (21% to 33% for women; 8% to 22% for men), and twenty-nine volunteers (49%) had high % body fat (>33% for women; >22% for men) (Gallagher et al., 2000) (**Table 1**).

Table 1. Characteristics of the volunteers participating in the study (*n* = 59).

Nutritional status	Sex	Age (years)	BMI[1] (kg/m^2)	%BF[2]
Normoweight (*n* = 24)	Women (*n* = 15)	31.1 ± 10.7	22.4 ± 2.1	28.4 ± 4.8
	Men (*n* = 9)	32.8 ± 5.2	23.4 ± 0.8	16.6 ± 5.2
	All volunteers	**32.4 ± 8.8[a]**	**22.8 ± 1.7[b]**	**23.7 ± 7.7[b]**
Overweight/Obese (*n* = 35)	Women (*n* = 16)	36.2 ± 7.7	30.2 ± 2.1	39.3 ± 3.8
	Men (*n* = 19)	31.9 ± 7.1	27.8 ± 0.8	20.9 ± 5.1
	All volunteers	**33.9 ± 7.6[a]**	**28.8 ± 2.8[a]**	**29.4 ± 10.3[a]**

[1]Body mass index; [2]Percentage body fat. Different superscript letters in the same column indicate significant differences between mean values of normoweight and overweight/obese volunteers (Mann-Whitney test, $p < 0.05$).

The study consisted of daily consumption of four capsules containing either the placebo (2 g of corn starch) or JPSP (3 g containing 35.2 mg of vescalagin + ellagic acid) for three periods of three weeks, in the following order: 1) placebo consumption; 2) JPSP consumption; 3) placebo consumption, and were instructed to store the flask with the capsules at room temperature. During all study periods, volunteers were instructed to maintain their habitual diet. To verify possible changes in the individuals' metabotype and in the amount of urolithins excreted, volunteers acutely ingested four capsules of JPSP right before the beginning of each period and after the last period. The total urine between 24 h and 36 h (maximum excretion of urolithins, according to Inada et al., 2019) after each acute ingestion of JPSP was collected (**Figure 1**). Total urine volume was determined, and samples were aliquoted and stored at -80 °C until analysis. From the four days before JPSP acute ingestion until the end of the urine collection, volunteers were instructed to restrict their consumption of ellagitannin food sources, which included red and black fruits (jaboticaba, strawberry, grape, açaí, cherry, pomegranate, cranberry, blackberry, blueberry) and their derived products (juices, soft drinks,

wines), nuts (walnuts, almonds, peanuts, hazelnuts) and their derived products, and black and green teas. Volunteers' compliance to food restrictions was confirmed during the study.

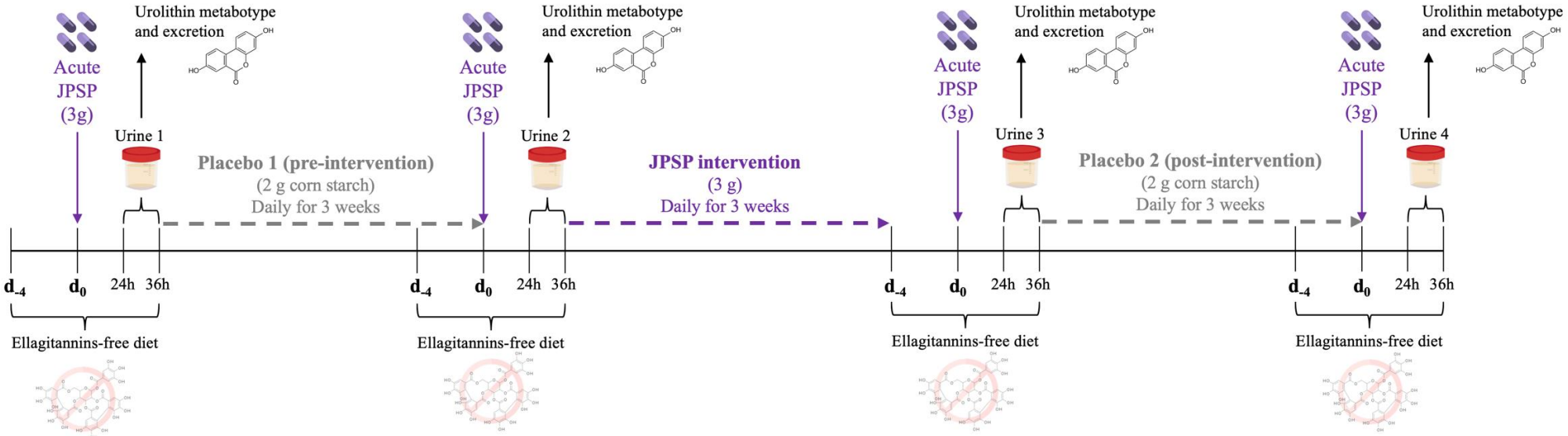

Figure 1. Illustrative diagram of the experimental design of the clinical study.

2.4 Urolithin metabolites analysis by HPLC-DAD-MS

Before the analysis, urine samples were centrifuged (4,696 × *g*, 10 min, 10 °C) and filtered with a 0.22 μm PVDF filter. The liquid chromatography system was the same as described in section 2.2, which was coupled to a LCMS-2020 quadrupole mass spectrometer with electrospray ionization (ESI). A N_2 generator (NM32LA, Peak Scientific®, Inchinnan, Renfrewshire) was used. Chromatographic separation of urolithins was performed according to García-Villalba et al. (2016). The stationary phase was a Poroshell 120 EC-C18 column (2.7 μm, 100 mm × 3 mm, Agilent Technologies), and the mobile phase consisted of a gradient of 0.5% aqueous formic acid (eluent A) and acetonitrile (eluent B) at a flow rate of 0.5 mL/min. Prior to injection, the column was equilibrated with 5% B. After injection of the sample, the solvent B composition increased to 18% B at 7 min, 28% B at 17 min, 50% B at 22 min, and 90% B at 27 min, which was maintained up to 28 min. Then, the initial conditions were re-established at 29 min and kept under isocratic conditions up to 33 min. The injection volume was 10 μL. Optimal ESI-MS parameters were as follows: capillary voltage of 3500 V; drying gas flow of 15 L/min; nebulizer gas flow of 1.5 L/min, desolvation temperature of 300 °C. MS spectra were acquired in negative ionization mode and measured in selective ion monitoring (SIM) mode. Urolithin A was identified by comparing its retention time, UV spectrum, and molecular mass to that of the authentic standard, and its quantification was performed by DAD (305 nm) using external standardization. Identification of other urolithins was carried out by their UV spectra and molecular masses, and their quantification was performed using the urolithin A calibration curve and their relative response factors according to García-Villalba et al., 2016.

2.5 Statistical analysis

Descriptive statistical analysis (mean, standard deviation, and coefficient of variation) was calculated using Microsoft® Excel® software (version 2304, 2019). The Shapiro-Wilk and Kolmogorov-Smirnov tests assessed data distribution, and medians were compared using the Mann-Whitney test. Excretion of urinary metabolites among collection points was compared using the Friedman test, followed by Dunn's multiple comparisons *post-hoc* test. A comparison of metabotypes' percentage was performed using the Chi-square test. Urolithin data were subjected to principal component analysis (PCA) to investigate similarities or differences among volunteers and the possible associations of variables. Statistical analyses were

performed using GraphPad Prism software for Windows, version 8.0.1 (GraphPad Software, San Diego, CA, USA). Results were considered significant when $p < 0.05$.

3. Results and discussion

3.1 Total phenolic compounds of JPSP are stable for 57 days of storage at room temperature

Five phenolic compounds were identified in JPSP: gallic acid, ellagic acid, vescalagin, delphinidin-3-*O*-glucoside, and cyanidin-3-*O*-glucoside. The total phenolic contents (sum of soluble and insoluble phenolics) of JPSP were 40.1 mg/g, of which insoluble phenolics represented 69% (**Table 2**). The higher content of insoluble compounds may be beneficial for modulating the intestinal microbiota and may be associated with a higher excretion of metabolites by the gut bacteria (Mosele et al., 2015).

Table 2. Soluble and insoluble phenolic compounds contents in jaboticaba peel and seed powder (JPSP)[1]

Phenolic compounds	**Content (mg/g)**
Soluble phenolics[2]	
Gallic acid	1.25 ± 0.00
Ellagic acid	2.06 ± 0.00
Vescalagin	8.47 ± 0.21
Delphinidin-3-*O*-glucoside	0.17 ± 0.00
Cyanidin-3-*O*-glucoside	0.55 ± 0.01
Soluble + insoluble phenolics[3]	
Gallic acid	4.87 ± 0.02
Ellagic acid	26.33 ± 1.49
Vescalagin	8.87 ± 0.10
Total soluble compounds	**12.50 ± 0.22**
Total soluble + insoluble compounds	**40.07 ± 1.51**
Total insoluble[4]	**27.57 ± 1.29**

[1]Results expressed in dry weight basis (dwb) as mean ± SD of three replicates. [2]Soluble compounds were determined after sample extraction with methanol 70%. [3]Soluble + insoluble compounds were determined after sample hydrolysis with HCl 4 N and extraction with methanol:DMSO (50:50, *v*/*v*). [4]Insoluble compounds were estimated as the difference between soluble + insoluble and soluble compounds contents.

Considering total phenolics, ellagic acid was the most abundant, representing 71% of the total, followed by vescalagin (22%). When the % of phenolic compounds is relative to only soluble compounds, their abundance shifted, with vescalagin being the most abundant (68%), followed by ellagic acid (17%) (**Table 2**). We did not observe castalagin in JPSP, which is an

ellagitannin that has been reported in jaboticaba. The contents of phenolic compounds in JPSP agree with those described for jaboticaba peel and seed powders, except for anthocyanins, which the contents were about eight times lower in JPSP (Inada et al., 2020a; Inada et al., 2019). While JPSP was obtained by oven-drying peel and seeds that were the residue of the production of a steam-extracted juice, the powders reported in the literature were produced by oven-drying a residue obtained by depulping the fruit. Therefore, the observed difference in the contents of anthocyanins, which are thermally unstable (Martins et al., 2021), could be explained by the use of an additional thermal process to produce the JPSP used in the present study.

The amount of insoluble phenolics in the jaboticaba peel and seed powder reported by Inada et al. (2019) was 62% of total phenolics, similar to the amount found in the present study (69%) (**Table 2**). Quatrin et al. (2019) evaluated the free and insoluble phenolics content of a freeze-dried jaboticaba peel powder, and the insoluble content was 15.8%. In the present study, the peel and the seeds were used to produce the powder, which contains higher amounts of insoluble phenolics, mainly tannins, than the peel (Inada et al., 2021).

Irrespective of storage temperatures (5 ^{o}C *vs.* 25 ^{o}C), total and individual phenolic contents did not change after 57 days of storage (**Figure 2**). This behavior could be explained by the low JPSP water activity, which slows chemical and enzymatic reactions and favors the preservation of phenolic compounds (Inada et al., 2019). This result allowed JPSP capsules to be stored at room temperature during the clinical study and, most importantly, guaranteed that volunteers ingested the same amount of phenolic compounds throughout the clinical trial.

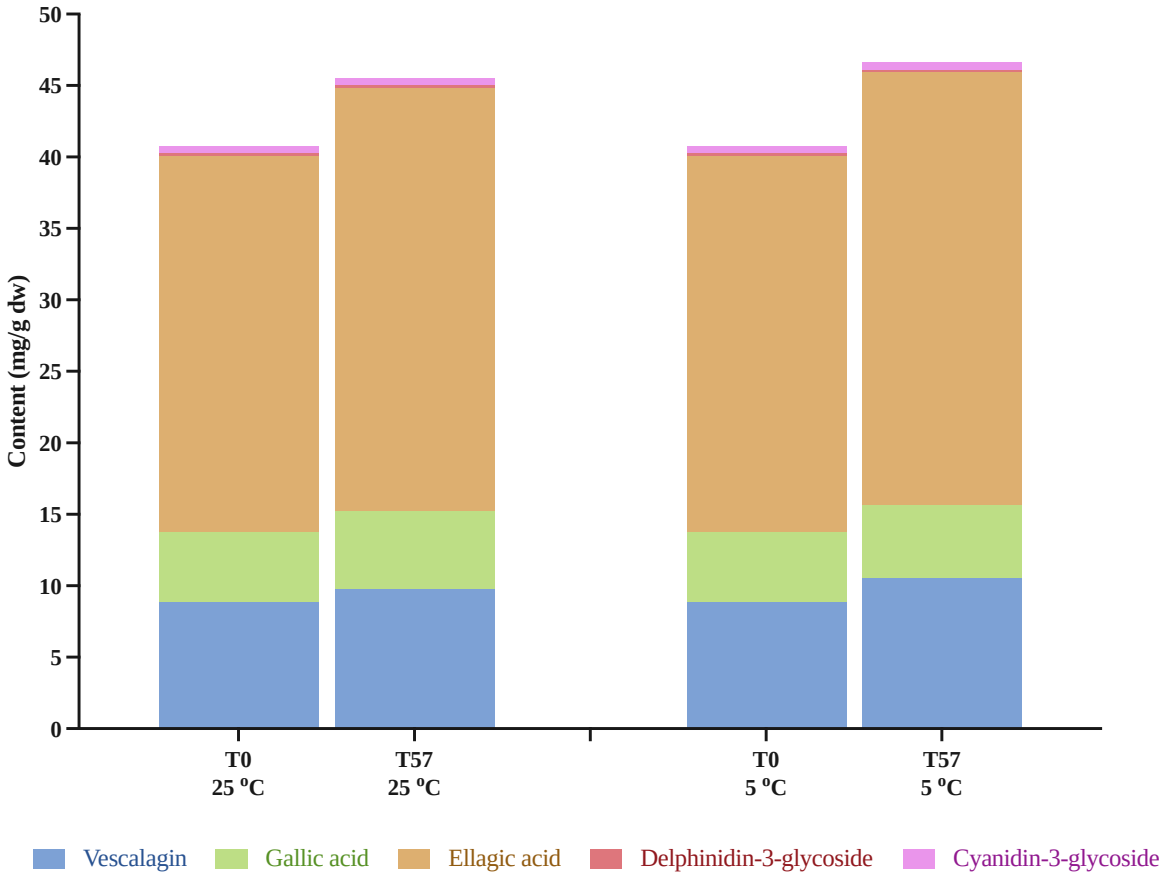

Figure 2. Contents of phenolic compounds in Jaboticaba peel and seed during storage at 25 ºC and 5 ºC. T0 = 0 days of storage; T57 = 57 days of storage. Neither temperature nor storage time caused changes in the content of phenolic compounds (two-way ANOVA, $p > 0.05$).

3.2 Volunteers showed shifts in their metabotypes during the clinical trial regardless of JPSP intervention

The amount of ellagitannins and derivatives (sum of vescalagin and ellagic acid) ingested daily by the volunteers in the present study (35.2 mg) is in accordance with many studies in the literature (García-Villalba et al., 2022; Tomás-Barberán et al., 2017), but much lower than that offered in a previous study from our research group (1,270 mg). This was done to avoid the laxative side effects observed (Inada et al., 2019). Nevertheless, classifying individuals into metabotypes was possible even when lower doses of ellagitannins (3.5 mg) were administered (Volpp et al., 2020).

After the acute ingestion of JPSP, seven urinary metabolites were identified: urolithin A 3/8-glucuronide (Uro-A 3/8-glu), isourolithin A 3-glucuronide (IsoUro-A 3-glu), isourolithin A 9-glucuronide (IsoUro-A 9-glu), urolithin A (Uro-A), isourolithin A (IsoUro-A), urolithin B-glucuronide (Uro-B-glu), and urolithin B (Uro-B) (**Table 3**). The

glucuronidated forms were the most abundant metabolites, representing 93% to 99% of the total excreted at each study stage. This was expected since phenolic compounds mainly undergo phase-2 metabolism, which involves glucuronidation and sulfation reactions in both the intestine and the liver (Romo-Vaquero et al., 2015; Truchado et al., 2012; Manach et al., 2004). Other authors also report that the main ellagitannin metabolites excreted in the urine are the glucuronides and sulfates of urolithins and, in smaller amounts, isourolithins (Ulaszewska et al., 2020; Cortés-Martín et al., 2018). A recent review consider that urolithins glucuronides are the most bioactive metabolites of ellagitannins (García-Villalba et al., 2022).

Table 3. Urinary excretion of urolithin metabolites (µmol) in the four urine samples collected during the clinical trial[1]

Metabolite	**Urine 1** **(before placebo 1)**	**Urine 2** **(before JPSP)**	**Urine 3** **(after JPSP)**	**Urine 4** **(after placebo 2)**
Uro-A 3/8-glu	1.17 ± 1.58[a] (*n* = 39; 66%)	1.06 ± 1.37 [a] (*n* = 39; 66%)	1.82 ± 2.84 [a] (*n* = 41; 70%)	1.15 ± 1.67[a] (*n* = 36; 61%)
IsoUro-A 3-glu	0.41 ± 0.86[a] (*n* = 21; 36%)	0.27 ± 0.59 [a] (*n* = 22; 37%)	0.34 ± 0.81[a] (*n* = 23; 39%)	0.35 ± 1.21[a] (*n* = 17; 29%)
IsoUro-A 9-glu	0.04 ± 0.17[a] (*n* = 4; 7%)	0.006 ± 0.03 [a] (*n* = 3; 5%)	0.002 ± 0.015 [a] (*n* = 2; 3%)	0.03 ± 0.20 [a] (*n* = 1; 2%)
IsoUro-A	0.008 ± 0.05[a] (*n* = 2; 3%)	0.012 ± 0.09 [a] (*n* = 1; 2%)	0.027 ± 0.16 [a] (*n* = 2; 3%)	0.014 ± 0.11 [a] (*n* = 1; 2%)
Uro-B-glu	0.60 ± 1.11[a] (*n* = 25; 42%)	0.62 ± 1.18 [a] (*n* = 22; 37%)	0.59 ± 1.23 [a] (*n* = 25; 42%)	0.64 ± 1.39[a] (*n* = 22; 37%)
Uro-B	0.016 ± 0.08[a] (*n* =3; 5%)	0.022 ± 0.09 [a] (*n* = 4; 7%)	0.005 ± 0.03 [a] (*n* = 2; 3%)	0.004 ± 0.02 [a] (*n* = 2; 3%)
Uro-A	0.005 ± 0.04[a] (*n* =1; 2%)	0.060 ± 0.41[a] (*n* = 2; 3%)	0.032 ± 0.18 [a] (*n* = 2; 3%)	0.039 ± 0.16 [a] (*n* = 4; 7%)
Total	**2.25 ± 2.29**	**2.11 ± 2.08**	**2.85 ± 3.51**	**2.27 ± 2.97**

[1]Results expressed as mean ± SD. No significant difference was found between the mean excretion of metabolites in the urine samples collected in the clinical trial. %: percentage of individuals who excreted the metabolite (Friedman test followed by Dunn's multiple comparison *post hoc* test, $p > 0.05$).

The volunteers were classified among the urolithin metabotypes by analyzing the four urine samples collected during the clinical trial (**Table 4**). Thirty-nine volunteers (66%) did not modify their metabotype during the clinical trial, especially those classified as UM-B, suggesting that this metabotype would be less susceptible to variation. Interestingly, many volunteers showed multiple metabotype shifts during the study, which were unrelated to sex, BMI, or % body fat. Shifts were also unrelated to JPSP intervention, as 13, 10, and 12 volunteers showed metabotype shifts when urine samples 1-2, 2-3, and 3-4 were compared, respectively. Metabotype shifts are apparently associated with changes in the amount of urolithins excreted

rather than changes in the production of different metabolites. In this regard, 27% of volunteers shifted from non-producers (UM-0) into producers (UM-A or UM-B) or vice-versa, and only 7% of volunteers shifted from UM-A into UM-B or vice-versa. Gonzalez-Sarrías et al. (2017) observed that the consumption of an ellagitannin-rich pomegranate extract for three weeks converted 50% of the individuals classified as non-producers (UM-0) into producers (either UM-A or UM-B). To the best of our knowledge, this is the first study to evaluate the urolithin metabotype of a given population at more than two moments. Considering our results, the stratification of individuals participating in a clinical trial according to their metabotype should not be performed only once but multiple times during the extent of the study. Other important measures to correctly identify metabotypes would be offering high doses of ellagitannins to volunteers and/or employing methods of higher sensitivity to analyze biological samples.

Table 4. Sex, body mass index (BMI), percentage body fat (%BF), and metabotype classification of volunteers.

Volunteer	Sex	BMI (kg/m^2)	%BF	Metabotype			
				Urine 1	Urine 2	Urine 3	Urine 4
1	Woman	21.3	23.3	B	B	B	B
2	Woman	24.7	33.0	B	B	B	B
3	Woman	21.6	32.6	A	A	0	A
4	Woman	24.7	35.7	A	A	A	0
5	Woman	18.0	22.7	A	A	0	0
6	Woman	23.3	27.1	B	B	B	B
7	Woman	23.2	29.6	B	B	B	B
8	Woman	23.3	24.8	0	0	0	0
9	Woman	20.9	20.9	B	0	B	B
10	Woman	23.7	32.8	B	B	B	B
11	Woman	22.1	23.2	B	B	B	B
12	Woman	24.8	34.0	B	B	B	B
13	Woman	18.8	28.4	B	B	B	B
14	Woman	21.4	25.6	B	B	B	B
15	Woman	24.0	33.0	B	B	B	B
16	Man	22.2	13.4	0	A	B	0
17	Man	24.6	23.5	B	B	B	B
18	Man	22.9	12.5	B	A	A	0
19	Man	23.3	25.2	B	B	B	B
20	Man	22.4	7.8	B	B	B	B
21	Man	23.3	17.5	B	B	B	B
22	Man	23.4	18.1	B	B	B	B
23	Man	24.6	16.9	B	B	B	B
24	Man	24.1	17.8	A	0	0	A
25	Woman	30.9	39.2	A	A	A	A
26	Woman	35.7	45.4	0	A	A	A
27	Woman	28.2	37.8	B	B	B	B
28	Woman	28.3	36.6	B	B	B	B

29	Woman	31.8	44.8	B	B	B	B
30	Woman	28.9	37.0	0	0	0	0
31	Woman	28.3	38.3	0	A	0	0
32	Woman	26.0	37.4	A	A	A	A
33	Woman	29.7	38.5	A	A	A	A
34	Woman	32.9	42.0	A	0	A	A
35	Woman	32.6	38.8	A	0	0	0
36	Woman	29.1	36.7	B	B	B	B
37	Woman	36.6	45.5	A	A	A	A
38	Woman	29.4	43.0	B	B	B	B
39	Woman	26.6	32.0	A	A	A	A
40	Woman	28.7	36.6	A	0	A	A
41	Man	29.0	23.7	B	B	B	0
42	Man	27.3	23.8	B	B	B	B
43	Man	29.3	28.9	A	A	A	A
44	Man	25.5	17.7	0	0	0	0
45	Man	27.5	15.0	B	B	0	B
46	Man	28.4	28.3	B	B	B	B
47	Man	29.3	27.1	B	B	B	B
48	Man	29.6	26.5	A	A	A	0
49	Man	30.9	19.7	B	B	B	B
50	Man	29.1	24.2	A	A	A	A
51	Man	31.5	20.4	A	A	A	A
52	Man	29.0	20.3	0	B	B	0
53	Man	26.3	19.0	0	A	0	A
54	Man	25.3	18.0	0	B	B	0
55	Man	26.0	14.8	B	0	A	A
56	Man	25.2	11.7	A	A	A	A
57	Man	25.3	12.7	A	A	B	A
58	Man	26.1	22.7	A	A	A	A
59	Man	25.4	23.5	B	B	B	B

3.3 Metabotype B was the most prevalent among volunteers, and metabotype distribution was influenced by BMI and age

Considering all volunteers (n = 59) and the four urine samples collected during the clinical trial, the percentage of UM-A was 32.2%, UM-B ranged from 45.8% to 54.2%, and UM-0 ranged from 13.6% to 22. % (**Figure 3**), with no significant differences during the trial (Chi-square test, $p > 0.05$). This result could lead to the incorrect conclusion that metabotypes would be less susceptible to variation. Still, as we already described in the previous section, that is most certainly not the case for these volunteers, reinforcing the importance of looking at individual metabotype data rather than its percentage. The observed percentage values differed from those reported by most studies with healthy individuals (**Table 1 of literature review**),

with a higher percentage of UM-A, followed by UM-B and UM-0. The distribution of metabotypes could be influenced by various parameters, such as nutritional status, age, and genetic background (Cortés-Martín et al., 2018; Tomás-Barberán et al., 2017).

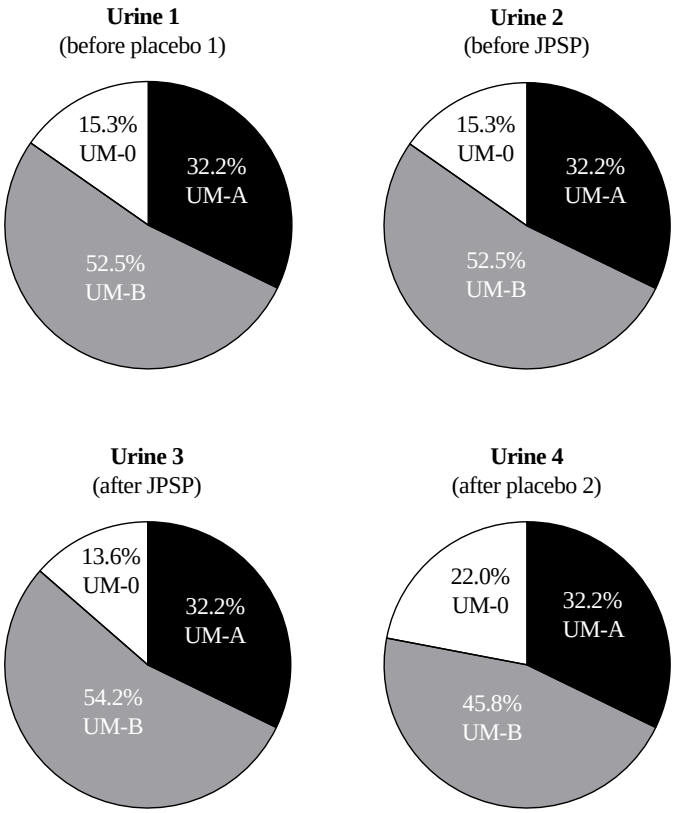

Figure 3. Percentage of urolithin metabotypes in the four different urine samples collected during the clinical trial. UM-A: metabotype A; UM-B: metabotype B; UM-0: metabotype 0. No statistical difference was observed in percentage values during the trial.

The percentage of UM-B has been associated with chronic diseases, such as metabolic syndrome and dysbiosis (Tomás-Barberán et al., 2014). Some studies have shown that UM-B individuals have a higher cardiovascular risk due to increased LDL-cholesterol values (Tomás-Barberán et al., 2014; González-Sarrías et al., 2010). However, a more recent study has shown that Uro-B-glucuronide concentration in urine was inversely correlated with LDL-cholesterol, a cardiovascular risk marker (Domínguez-López et al., 2023). One could argue that the association between UM-B and obesity is not convincing and straightforward, probably due to the multifactorial nature of this disease (Laveriano-Santos et al., 2022; Romo-Vaquero et al., 2019).

For women (n = 31), the percentage of UM-A ranged from 29.0% to 35.5%, UM-B from 48.4% to 51.6%, and UM-0 from 12.9% to 19.4%. For men (n = 28), the percentage of UM-A ranged from 28.6% to 25.7%, of UM-B from 39.3% to 57.1%, and of UM-0 from 10.7 % to 25.0 %. Independently of the period of the study (urine sample), no statistical difference was found when comparing metabotype percentage values between sexes (Chi-square test, p > 0.05), as already reported in the literature (Inada et al., 2019; Cortés-Martín et al., 2018). However, more recently, a study carried out with healthy NW Asian young adults (n = 35) reported different UM-B distributions between men (40%) and women (25%), although a similar distribution of UM-A (54%) was observed (Xian et al., 2021).

For NW volunteers (n = 24), the percentage of UM-A ranged from 12.5% to 16.7%, of UM-B from 66.7% to 75.0%, and of UM-0 from 8.3% to 20.8% (**Figure 4A**). For OW/OB volunteers (n = 35), the percentage of UM-A ranged from 42.9% to 45.7%, of UM-B from 31.4% to 40.0%, and of UM-0 from 14.3% to 22.9% (**Figure 4B**). Independently of the urine sample, the distribution of metabotypes differed between NW and OW/OB volunteers (p < 0.05). Even though differences in metabotype distribution according to nutritional status have already been reported (Cortés-Martín et al., 2018; Inada et al., 2019), most studies reported a higher percentage of UM-B in OW/OB individuals compared to NW, the opposite of what it was observed in the present study. Another exception is the data reported by Araujo et al. (2021), who observed a similar percentage for UM-B between NW (44%) and OW/OB individuals (42%).

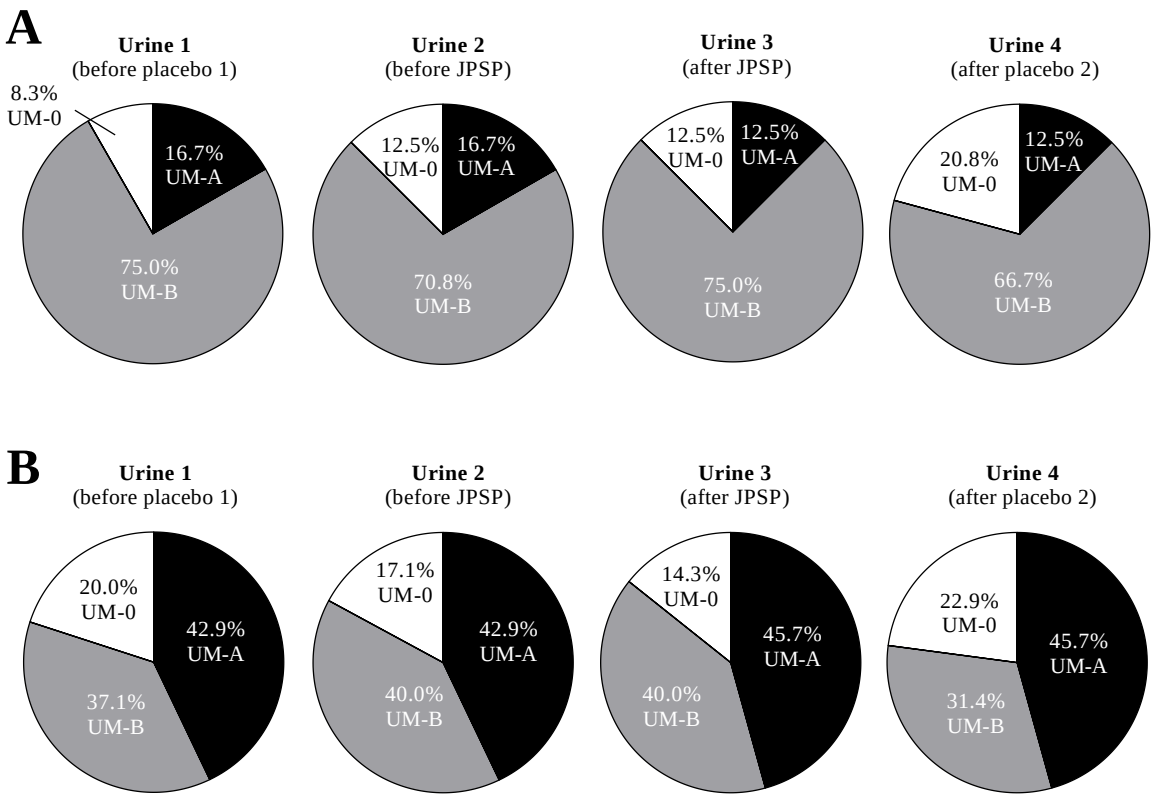

Figure 4. Percentage of urolithin metabotypes in the four different urine samples collected during the clinical trial in NW (**A**) and OW/OB (**B**) volunteers. UM-A: metabotype A; UM-B: metabotype B; UM-0: metabotype 0. Statistical difference was observed in percentage values during the trial between NW and OW/OB volunteers (Chi-square test, $p < 0.05$).

For an adequate % body fat volunteers ($n = 30$), the percentage of UM-A ranged from 16.7% to 30.0%, of UM-B from 53.3% to 60.0%, and of UM-0 from 16.7% to 23.3%. For high % body fat volunteers ($n = 29$), the percentage of UM-A ranged from 34.5% to 41.4%, of UM-B from 44.8% to 48.3%, and of UM-0 from 10.3% to 20.7%. For both classifications of % body fat, UM-B was more prevalent, differently from that observed when volunteers were classified according to BMI. Moreover, a significant difference in the distribution of metabotypes was observed between adequate % body fat (**Figure 5A**) and high % body fat (**Figure 5B**) ($p < 0.05$). However, this difference was only observed after JPSP intervention (urine 3), whereas differences were observed during the whole clinical trial when BMI was considered. To the best of our knowledge, our study is the first to evaluate metabotype distribution according to volunteers' %BF.

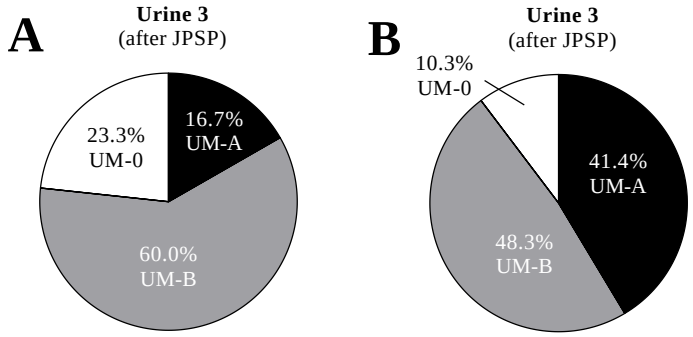

Figure 5. Percentage of urolithin metabotypes after JPSP consumption (urine 3) in adequate % body fat (**A**) and high % body fat (**B**) volunteers. UM-A: metabotype A; UM-B: metabotype B; UM-0: metabotype 0. Statistical difference was observed between in adequate % body fat and high % body fat (Chi-square test trend, $p < 0.05$).

For volunteers between 19 to 30 years old ($n = 28$), the percentage of UM-A ranged from 39.3% to 50.0%, of UM-B from 39.3% to 42.9%, and of UM-0 from 10.7% to 21.4% (**Figure 6A**). For volunteers between 31 to 40 years old ($n = 19$), the percentage of UM-A ranged from 26.3% to 31.8%, of UM-B from 42.1% to 52.6%, and of UM-0 from 10.5% to 26.3 % (**Figure 6B**). For volunteers older than 40 years old ($n = 12$), the percentage of UM-A ranged from 8.3% to 16.7%, of UM-B from 75% to 83.3%, and of UM-0 from 0% to 16.7% (**Figure 6C**). Independently of the urine sample, the distribution of metabotypes differed among age groups ($p < 0.05$). Cortés-Martín et al. (2018) reported that aging is related to an increasing percentage of UM-B (from 8% for 5-10-year-olds to 40% for 40-90-year-olds) and a decreasing percentage of UM-A (from 81% for 5-10-year-olds to 50% for 40-90-year-olds). Our volunteers were 33 ± 8 years old, which could explain, at least in part, the higher percentage of UM-B compared to the literature. The high percentage of UM-B observed by Araújo et al. (2021) was also argued to be partially related to the age of their volunteers (45 ± 11 years). Considering these findings, age would be an important bias in clinical trials that classify individuals according to their metabotypes. Therefore, one should recruit volunteers of similar age when performing such clinical studies.

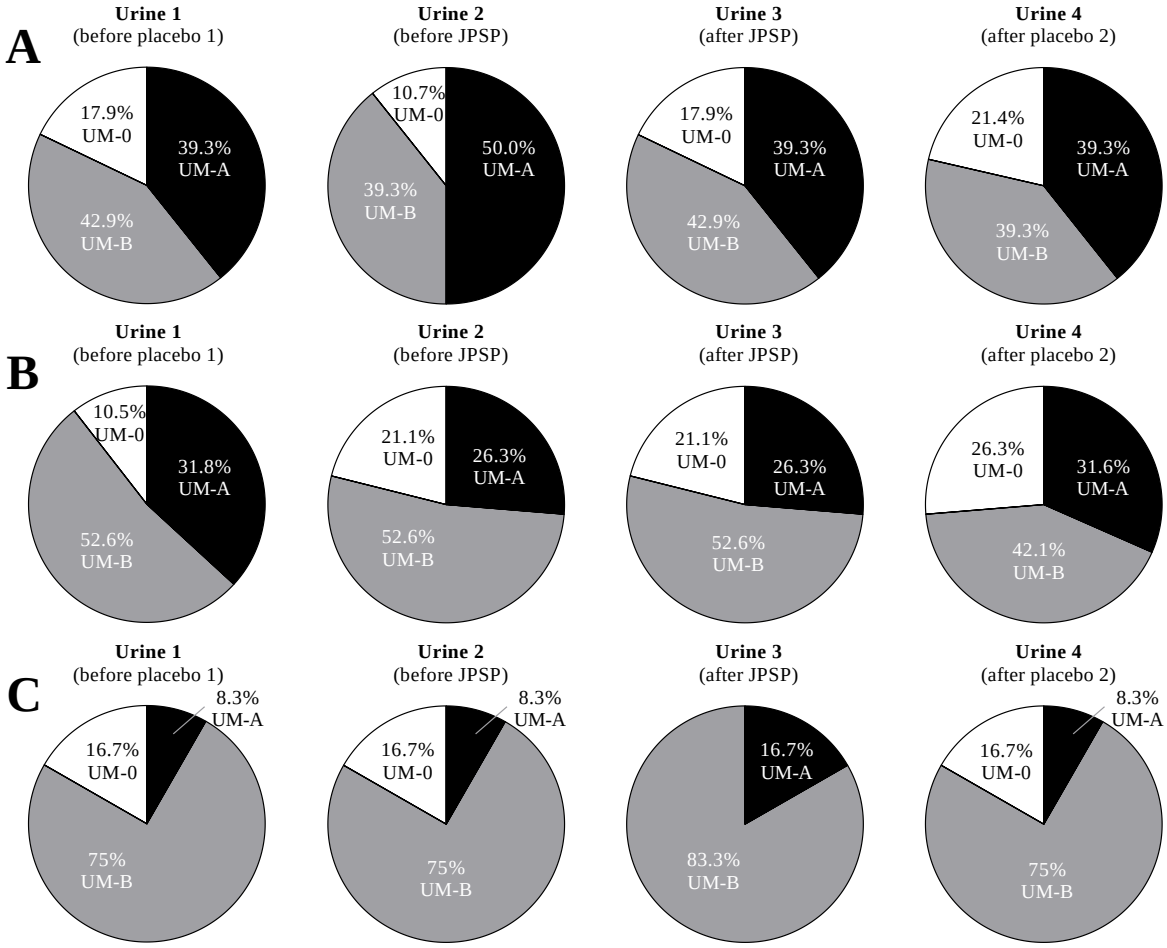

Figure 6. Percentage of urolithin metabotypes in the four different urine samples collected during the clinical trial in volunteers aged 19-30 years old (**A**), 31-40 years old (**B**), and older than 40 years old (**C**). UM-A: metabotype A; UM-B: metabotype B; UM-0: metabotype 0. Statistical difference was observed in percentage values during the trial among volunteers of different age groups (Chi-square test, $p < 0.05$).

3.4 Consumption of JPSP increased urolithin excretion in normoweight volunteers.

The amount of total urolithins excreted by volunteers ranged from 0.01 µmol to 9.41 µmol in urine 1, from 0.16 µmol to 8.24 µmol in urine 2, from 0.06 µmol to 15.39 µmol in urine 3, and from 0.1 µmol to 13.12 µmol in urine 4 (**Figure 7**), which is in accordance with the literature (García-Villalba et al., 2022; Inada et al., 2021). No significant differences were observed for the average excretion of total urolithins among the urine samples collected during the clinical trial ($p > 0.05$), possibly due to the high interindividual variability of the data, an aspect frequently observed in clinical studies addressing the metabolism of polyphenols.

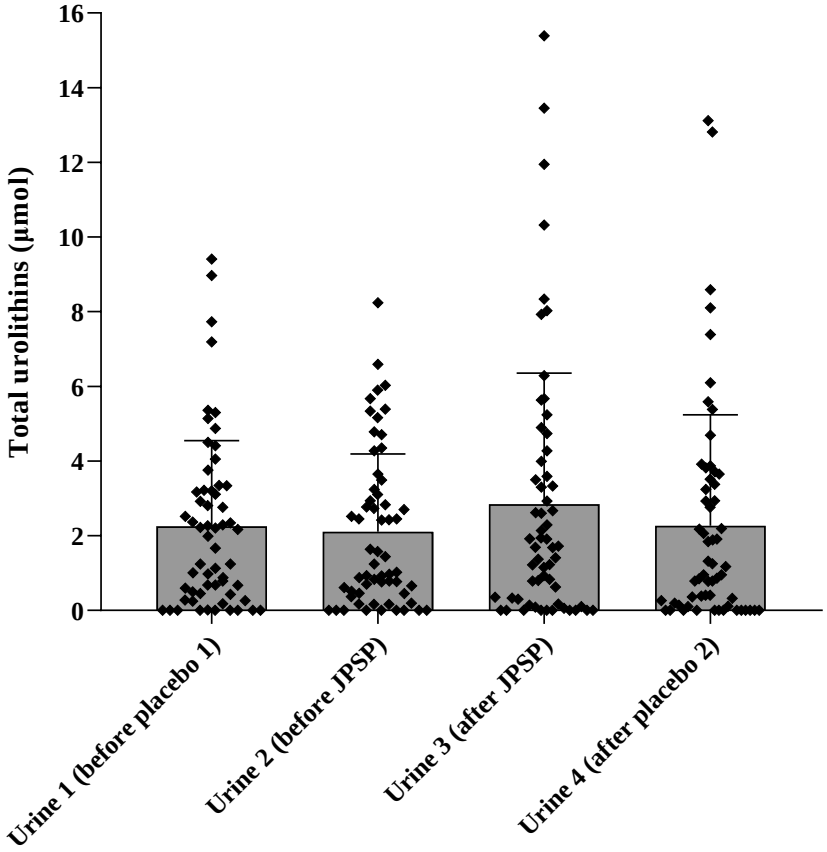

Figure 7. Urinary excretion of total urolithins (µmol) in the four urine samples collected during the clinical trial. No significant difference was observed among urine samples (Friedman test, $p > 0.05$).

Supposing a lower interindividual variability among volunteers with the same metabotype, we compared the urinary excretion of total urolithins by UM-A ($n = 21$) and UM-B volunteers ($n = 31$) that did not shift their metabotypes from UM-A to UM-B (and vice versa) during the whole clinical trial (**Figure 8**). Nonetheless, no significant differences were observed (Friedman test, $p > 0.05$).

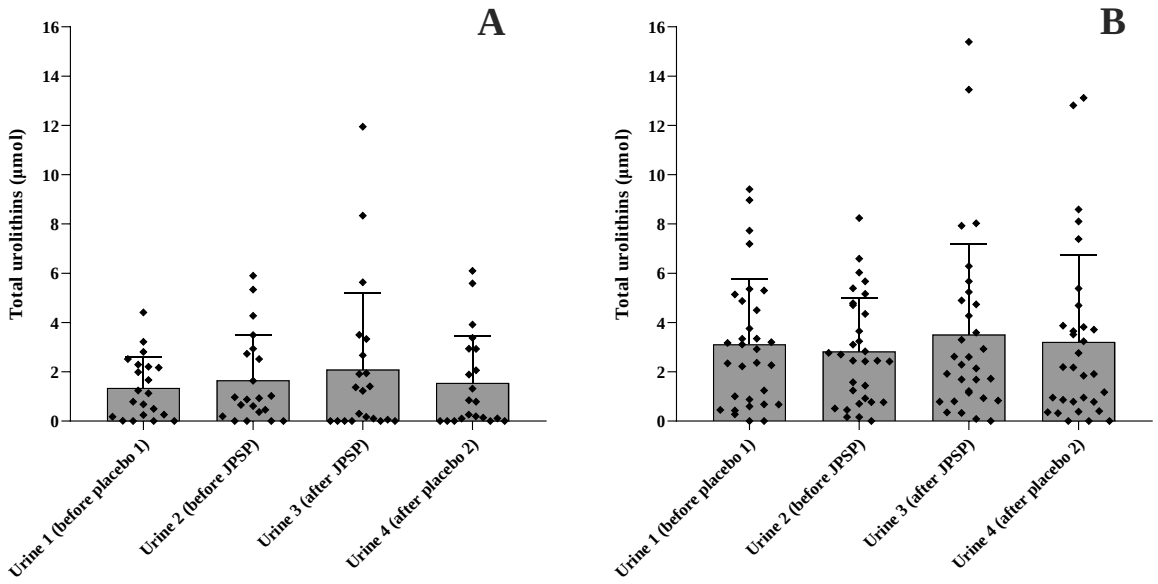

Figure 8. Urinary excretion of urolithin metabolites (µmol) in the four different urine samples collected during the clinical trial in UM-A volunteers (**A**) and UM-B volunteers (**B**) that did not shift their metabotypes from UM-A to UM-B (and vice versa).

We calculated the difference between the urinary excretion of a given urine sample and its previous one to investigate if the placebo interventions (*i.e.* urine 2 minus 1 and urine 4 minus urine 3) or the JPSP intervention (*i.e.* urine 3 minus urine 2) had any effects on urolithins excretion considering sex, BMI and % body fat. No significant effect of placebo and JPSP interventions was found when volunteers were grouped according to sex and % body fat. When volunteers were grouped according to BMI, we observed that the JPSP intervention, but not the placebo, led to an increase ($p = 0.0399$) in the excretion of total urolithins in NW volunteers. In contrast, the excretion in OW/OB volunteers did not change (**Figure 9**). This increase could be related to a modulation of the microbiota of these volunteers caused by the ingestion of JPSP for three weeks, suggesting that ellagitannins could have acted as prebiotics. In a study with overweight/obese patients with Barrett's esophagus, no differences in urinary excretion were observed between 12 and 26 weeks of daily consumption of black raspberry powder (Kresty et al., 2016). González-Sarrías et al., (2017b) also did not observe any effect of the extent of an intervention with a pomegranate extract on the excretion of urolithins by overweight/obese volunteers. Even though there are other studies in the literature dealing with chronic exposure to different doses of ellagitannins (25 mg to 789 mg) (Tulipani et al., 2012; Puupponen-Pimiä et al., 2013; Li et al., 2015), the effect of the intervention time on the urinary excretion of urolithins was not investigated.

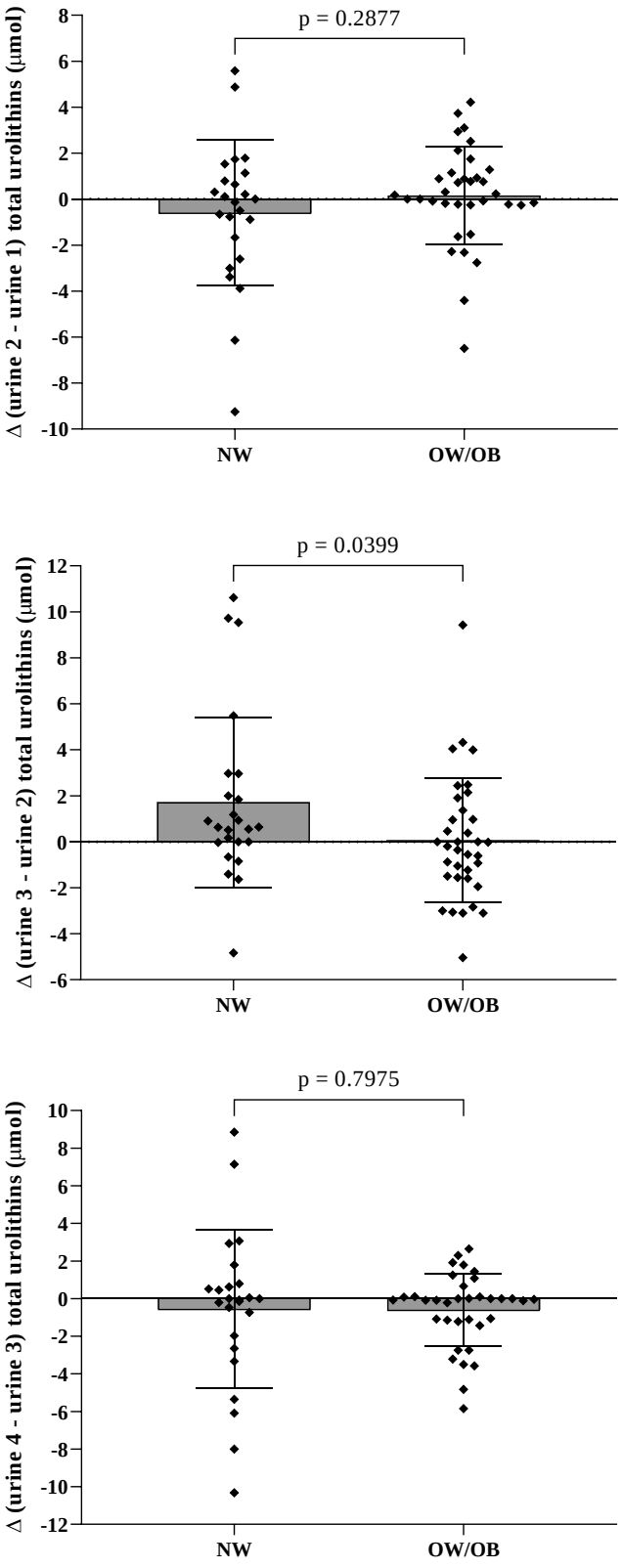

Figure 9. Change in total urolithin excretion due to interventions with placebo 1 (**A**), JPSP (**B**), and placebo 2 (**C**) between normoweight (NW) and overweight/obese (OW/OB) volunteers.

To investigate whether metabolite profiles would be associated with the characteristics of volunteers, we performed PCA analysis with the data. The first two principal components accounted for 20.4% and 18.0% of the variance in the plot, respectively. The metabolites with the highest contribution to the variability shown in the loadings plot were IsoUroA 9-glu, IsoUroA 3-glu, Uro-B-glu, and Uro-B (**Figure 10A**). Metabolites found in few volunteers, such as Uro-A (n = 2) and IsoUro-A (n = 1), as well as those found in most volunteers (Uro-A 3/8-glu, n = 39), did not contribute much to the variance of the data. Moreover, there seems to be more NW than OW/OB volunteers clustered in the lower-left quadrant of the PC scores plot (**Figure 10B**), indicating that the nutritional status of NW subjects would be associated with a higher excretion of Uro-B and Uro-B-glu.

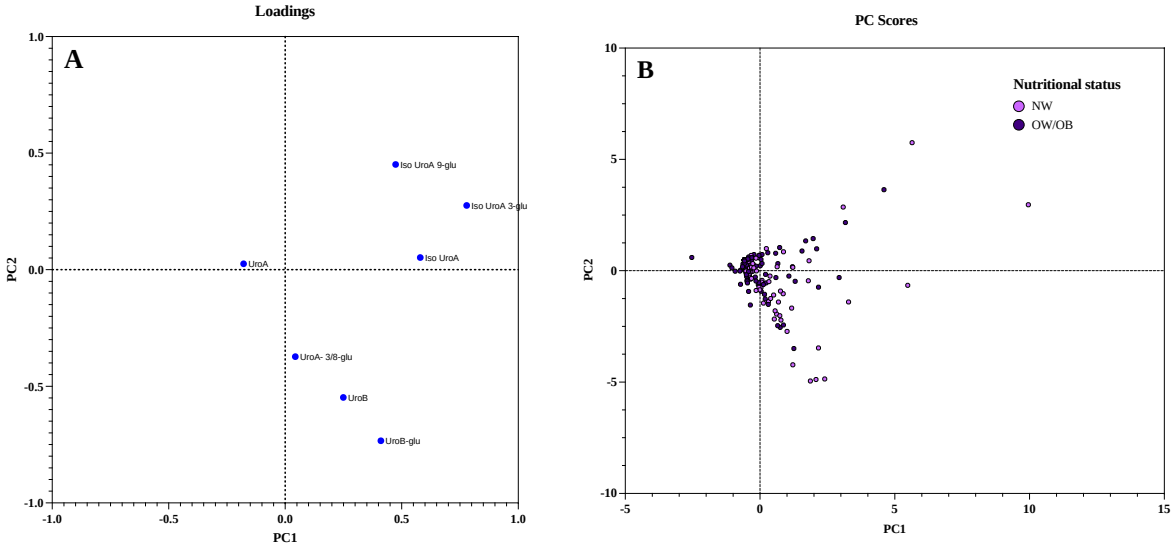

Figure 10. Loadings and PC score plots of urinary urolithin metabolites excreted by normoweight (NW) and overweight/obese (OW/OB) volunteers.

4. Conclusion

This is the first study that investigated ellagitannins metabolism after three weeks of the consumption of jaboticaba by Brazilian subjects. We showed that ellagitannins from jaboticaba peel and seeds powder were stable for 57 days when stored at room temperature and, therefore, could be consumed by the volunteers during the clinical trial.

Metabotype shifts were observed during the clinical study regardless of the intervention, and metabotype B seemed less susceptible to variation. This metabotype was more prevalent regardless of age, sex, and body fat percentage. Considering nutritional status, metabotype B was more prevalent only in normoweight volunteers, whereas metabotype A was

more prevalent in overweight/obese volunteers. Age also influenced the distribution of metabotypes, with increasing age being related to an increasing percentage of metabotype B.

Total urolithin excretion did not change during the clinical trial, even when volunteers were classified according to metabotypes A and B. However, when volunteers were grouped according to body mass index, we observed that the intervention with jaboticaba peel and seeds powder increased the excretion of total urolithins only by normoweight volunteers.

Conflict of interest

The authors declared that there is no conflict of interest.

Acknowledgments

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Future Perspectives of this Thesis

- The human health benefits associated with urolithins explain the relevance of identifying the responsible gut bacteria potentially helpful in developing novel probiotics, functional foods, and food complements. This is especially relevant in UM-0 individuals cannot produce bioactive urolithins or produce it at a non-detectable level.
- More studies to evaluate the metabotype in different periods should be carried out with a larger number of volunteers, especially with similar ages, to minimize the interindividual variation.
- The investigation of the effect of the chronic consumption of jaboticaba peel and seed powder on cardiometabolic markers such as total cholesterol, triglycerides, Apo A, and Apo B is important to evaluate the effect of a Brazilian berry on human health.

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Annex 1 - Consent Form

Universidade Federal do Rio de Janeiro – UFRJ
Centro de Ciências da Saúde – CCS
Instituto de Nutrição Josué de Castro – INJC

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO (TCLE)

Metabolismo de elagitaninos da farinha de jabuticaba em adultos

Você está sendo convidado(a) a participar de uma pesquisa cujo objetivo é estudar as mudanças que componentes naturalmente presentes na jabuticaba (compostos fenólicos) sofrem no corpo de seres humanos. Esse estudo é importante para entender melhor as ações da jabuticaba na saúde. Este estudo está sendo realizado pela nutricionista e doutoranda do Programa de Pós-graduação em Nutrição Iris Batista Leite, sob orientação dos professores Dr.ª Mariana Costa Monteiro da Fonseca e Dr. Daniel Perrone Moreira e da pesquisadora Kim Ohanna Pimenta Inada.

Sua participação consistirá em consumir farinha de jabuticaba. Participando desse estudo, quatro dias antes e durante a coleta de urina você será orientado a:

- Beber 2 litros de água por dia;

- Anotar todas as bebidas e alimentos consumidos;

- Não consumir oleaginosas (exemplo: nozes, castanhas, amendoim), cacau, chá, jabuticaba, romã.

O estudo será dividido em três partes. Você irá consumir, em três momentos, ao longo de 3 semanas cápsulas contendo placebo (amido) ou farinha de jabuticaba. No início e no final de cada intervenção (4 momentos) você voltará ao laboratório para consumir 4 cápsulas de farinha. Amostras de urina serão coletadas antes e no intervalo de 24-36 horas após engolir as cápsulas e no dia seguinte consumirá por 3 semanas cápsulas que poderão ser o placebo ou a farinha de jabuticaba. Todos os dados fornecidos são considerados confidenciais, garantindo totalmente a sua privacidade. Os dados não serão divulgados de forma a possibilitar a sua identificação. Os riscos envolvidos são mínimos que envolvem somente possíveis casos de reação alérgica aos pós de jabuticaba. Para prevenir esses casos, será utilizado como critério de exclusão dos voluntários a existência de qualquer tipo de alergia alimentar. Não há risco durante

o procedimento de coleta de amostras de urina e fezes, pois o mesmo não é invasivo. Faz-se necessária a lavagem das mãos com água e sabão após a coleta de urina para não adquirir ou transmitir nenhuma doença. Esclarecemos, ainda, que não há benefício direto para você, mas que sua participação contribui para complementar as informações na comunidade científica. Você será reembolsado de despesas relacionadas à pesquisa, tais como alimentação e transporte necessários para sua participação no estudo. A sua participação na pesquisa não é obrigatória e sua recusa não acarretará nenhum prejuízo. Além disso, você poderá se retirar da pesquisa a qualquer momento, sem qualquer tipo de aborrecimento, sem que isso lhe traga qualquer prejuízo ou punição, sem necessidade de justificativa.

Caso você venha a sofrer qualquer tipo de dano resultante de sua participação na pesquisa, previsto ou não neste Termo de Consentimento Livre e Esclarecido, você terá direito à indenização por parte do pesquisador, do patrocinador e das instituições envolvidas nas diferentes fases da pesquisa. Cabe enfatizar que a questão da indenização não é prerrogativa da Resolução CNS N° 466 de 2012, estando originalmente prevista no Código Civil (Lei 10.406 de 2002), sobretudo nos artigos 927 a 954, dos Capítulos I (Da Obrigação de Indenizar) e II (Da I (Da Obrigação de Indenizar), Título IX (Da Responsabilidade Civil).

Em caso de dúvidas ou questionamentos, você pode se manifestar agora ou em qualquer momento do estudo para explicações adicionais. Você terá acesso direto com os responsáveis pela pesquisa das seguintes formas:

Prof.ª Drª. Mariana Costa Monteiro da Fonseca E-mail:mariana@nutricao.ufrj.br
Telefone: 99961-5219
Endereço: Avenida Carlos Chagas Filho, 373. CCS, Bloco J, sala J2-16
Prof. Dr. Daniel Perrone Moreira E-mail: danielperrone@iq.ufrj.br
Telefone: 3938-7351
Endereço: Avenida Athos da Silveira Ramos, 149. Centro de Tecnologia, Bloco A, Sala 528A
Pesquisadora Kim Ohanna Pimenta Inada E-mail: kiminada@gmail.com
Telefone: 99635-0151
Doutoranda: Iris Batista Leite E-mail: irisbleite@gmail.com
Telefone: 98705-0096

Se você tiver alguma consideração ou dúvida sobre a ética da pesquisa, entre em contato com o Comitê de Ética em Pesquisa (CEP) do Hospital Universitário Clementino Fraga

Filho/HUCFF/UFRJ. O CEP é um órgão institucional que tem por objetivo proteger o bem-estar dos participantes de um estudo clínico por meio da avaliação dos projetos de pesquisa que envolvam a participação de seres humanos. O CEP está localizado na Rua Professor Rodolpho Paulo Rocco, nº 255, 7º andar, Ala E. Cidade Universitária/Ilha do Fundão. Rio de Janeiro. Telefone (21) 3938-2480, atendimento de segunda a sexta-feira, das 8 às 16 horas ou através do email: cep@hucff.ufrj.br.

Li e concordo em participar da pesquisa. Eu receberei uma via desse Termo de Consentimento Livre e Esclarecido (TCLE) e a outra ficará com o pesquisador responsável por essa pesquisa. Além disso, estou ciente de que eu e o pesquisador responsável deveremos rubricar todas as folhas desse TCLE e assinar na última folha.

_____ _____

Data: ____/____/20__

Nome do participante da Pesquisa Assinatura do participante da Pesquisa

_____ _____

Data: ____ /___/20__

Nome do Pesquisador Responsável Assinatura do Pesquisador Responsável