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Chapter IV

Dietary Isoflavones and Intestinal Microbiota: Metabolism and Transformation into Bioactive Compounds

*Maddalena Rossi**, *Alberto Amaretti*, *Lucia Roncaglia*,
Alan Leonardi and *Stefano Raimondi*
Dept. Chemistry, University of Modena and Reggio Emilia, Italy

Abstract

Edible plants provide the human with hundreds of non-nutritional phytochemicals which are recognized as beneficial, such as isoflavones. Likewise other polyphenols, isoflavones may undergo extensive transformations during passage through human digestive tract, especially in the colon, where members of the complex commensal microbiota are capable to carry out synergistically a broad range of metabolic transformations affecting the fate and the biological activity of phytochemicals. Diverse bacterial species occurring in the large intestine hydrolyze the glucose conjugated forms of isoflavones, releasing the corresponding aglycones, which may undergo further microbial conversions (especially reductions) giving rise to a wide spectrum of isoflavone-derived compounds. The end-products of microbial transformations are subjected to substantial person-to-person variation, reflecting the impact of the colonic microbiota, since intestinal bacteria may greatly increase or compromise the biological activity of dietary isoflavones. In fact, certain reduction products (e.g. S-equol) possess superior antioxidant and estrogen-like activities, while others are precursors for C-ring cleavage and lead to isoflavone degradation. Extensive research has been performed to characterize the intestinal bacteria which are responsible for isoflavones transformations. In particular, many efforts are being carried out to identify single bacteria that convert the

* Corresponding author: Dept. of Chemistry, University of Modena and Reggio Emilia, via Campi 183, 41125, Modena, Italy, tel. +39-059-2055567, fax. +39-059-373543, E-mail: maddalena.rossi@unimore.it

major soy isoflavone daidzein into S-equol, in the perspective to enrich soy products with such a valuable isoflavone-derived metabolite. Besides, diverse bacteria which are capable of deglycosylation, demethylation, and various reduction reactions are increasingly being identified. Nonetheless, only bifidobacteria and lactobacilli, which produce β -glucosidase that hydrolyze isoflavone glycosides into the corresponding aglycones, are currently exploited to improve the biological activity of soymilk.

Introduction

Edible plants are dietary sources of hundreds of non-nutritional phytochemicals. These molecules are typically secondary metabolites and occur in small quantities in plant tissues and plant-derived foods. The major classes of bioactive phytochemicals are polyphenols (which include phenolic acids, flavonoids, stilbenes, lignans, and tannins), organosulphur compounds (which include glucosinolates and isothiocyanates), carotenoids, plant sterols, monoterpenes, and alkaloids (Kris-Etherton et al. 2002). A number of beneficial activities, including antioxidant, antitumoral, and estrogenic-like properties, have been demonstrated for thousands of diverse phytochemicals through numerous epidemiologic, clinical, and experimental studies (Cederroth and Nef 2009, Crozier et al. 2009).

The level of bioactive phytochemicals within the body is largely determined by diverse phenomena, such as the digestive transformation of native compounds, the absorption in the intestine, the hepatic activity, and the biliary or urinary excretion (Scalbert and Williamson 2000, Manach et al. 2004). The phytochemicals which are not absorbed in the small intestine reach the colon, where they may undergo extensive biotransformation by the resident microbiota (Manach et al. 2004, Aura 2008, Selma et al 2009). The bacterial transformation may lead to the inactivation and/or degradation of phytochemicals or may cause the production of compounds with enhanced biological activity or bioavailability. Examples of the specific conversion of diverse molecules into bioactive metabolites accomplished by the microbiota are the conversion of lignans into enterolactone and enterodiols and the conversion of soy isoflavones into S-equol (Clavel et al. 2006a, Clavel et al. 2006b, Tsangalis et al 2007, Wang et al. 2007). During the course of absorption, phytochemicals are conjugated in the intestine and later in the liver, being subject to methylation, sulfation, and β -glucuronidation. Then, they can be secreted in the duodenum with the bile as hydrophilic conjugates. The colonic microbiota is involved in the enterohepatic recycling of phytochemicals. In fact, the microbial enzymes, especially β -glucuronidase, can deconjugate the excreted phytochemicals in the colon, where they can be reabsorbed, leading to a longer presence in the body (Manach et al. 2004, Zhang et al. 2007).

Likewise many other phytochemicals, isoflavones are converted by host enzymes and mostly by bacteria of the human intestinal microbiota. Bacterial transformations are essential for their absorption, bioavailability, and estrogenic properties. *In vivo* studies demonstrated that variations in the activity of isoflavones occur among different subjects, this interindividual variation being mainly attributed to differences in the composition of the gut microbiota (Xu et al. 1995, Zhang et al. 1999, Hur et al. 2002).

The mammalian gut is one of the most densely populated ecosystems on Earth. In particular, the human large intestine is colonized by a complex community composed largely of anaerobic bacteria, whose cell numbers exceed 10^{11} per gram of intestinal content. The

adult human gastrointestinal tract contains all three domains of life (bacteria, archaea, and eukarya), but it is predominated by bacteria belonging to *Firmicutes*, *Bacteroidetes* and *Actinobacteria* (Eckburg et al. 2005) (Figure 1). *Firmicutes* are by far the most abundant and diverse group, and include the *Clostridia* and *Bacilli* class; *Bacteroidetes* are also present in high numbers. This vast microbiota fulfils its energy needs using, through hydrolytic and fermentative pathways, various types of substrates left undigested and unabsorbed in the small intestine. Oligo- and polysaccharides, proteins and peptides are broken down by bacterial enzymes to their oligomeric and/or monomeric components. Then, sugars and amino acids are fermented, yielding organic acids (such as lactic, propionic, and butyric acids), branched chain fatty acids (such as isobutyric, isovaleric, and 2-methylbutyric acids), H₂, CO₂, ammonia, amines and several other end-products. Short chain fatty acids (SCFA) are, from a nutritional point of view, the major fermentation products. Butyrate is of particular importance because it is the major energy source for colonocytes, the epithelial cells that line the colon. SCFA affect the metabolism, growth, and differentiation of colonocytes, influence the hepatic control of lipids and carbohydrates, and provide muscle, kidney, heart and brain with energy (O'Keefe 2008).

In the mutualistic relationship between mammals and commensal intestinal bacteria, the bacterial metabolism and the cross-talk between bacterial and animal cells have a major impact on the nutrition and the overall health status of the host. Microorganisms are provided with a broad spectrum of fermentable compounds and a temperature controlled anoxic environment, while the host gains the protection against infections, the modulation of the immune system and the supply of carbon, energy, vitamins, and bacterial-activated dietary metabolites, including isoflavones (Leser and Mølbak 2009, O'Keefe et al. 2009).

In this chapter, it is discussed the role of intestinal bacteria in transforming the major isoflavones occurring in diet. Attention is focused on the microbial hydrolysis of isoflavone glycoconjugates, on the reactions which transform the aglycones into molecules with enhanced biological activity or, conversely, into inactive compounds, and on the bacterial groups which are responsible for such transformations. Most information is presented for soybean isoflavones (daidzein, genistein, and glycitein and their corresponding glycoconjugates), which have received the greatest interest and for which plenty of scientific literature is available. Furthermore, what is known about the bacterial transformations of other less common isoflavones, such as puerarin and the red clover isoflavones formononetin and biochanin A is described.

Hydrolysis of Isoflavone Glycoconjugates

Even though the diet can provide the human with beneficial isoflavones, their bioavailability and biological activity are largely determined by the form in which they occur. In food, and especially in soy-based products, the aglycone forms of isoflavones (e.g. daidzein, genistein and glycitein) are generally less abundant than the respective glycoconjugates, which include glucosides (daidzin, genistin and glycitin), acetylglucosides (acetyldaidzin, acetylgenistin and acetylglycitin), and malonylglucosides (malonyldaidzin, malonylgenistin and malonylglycitin).

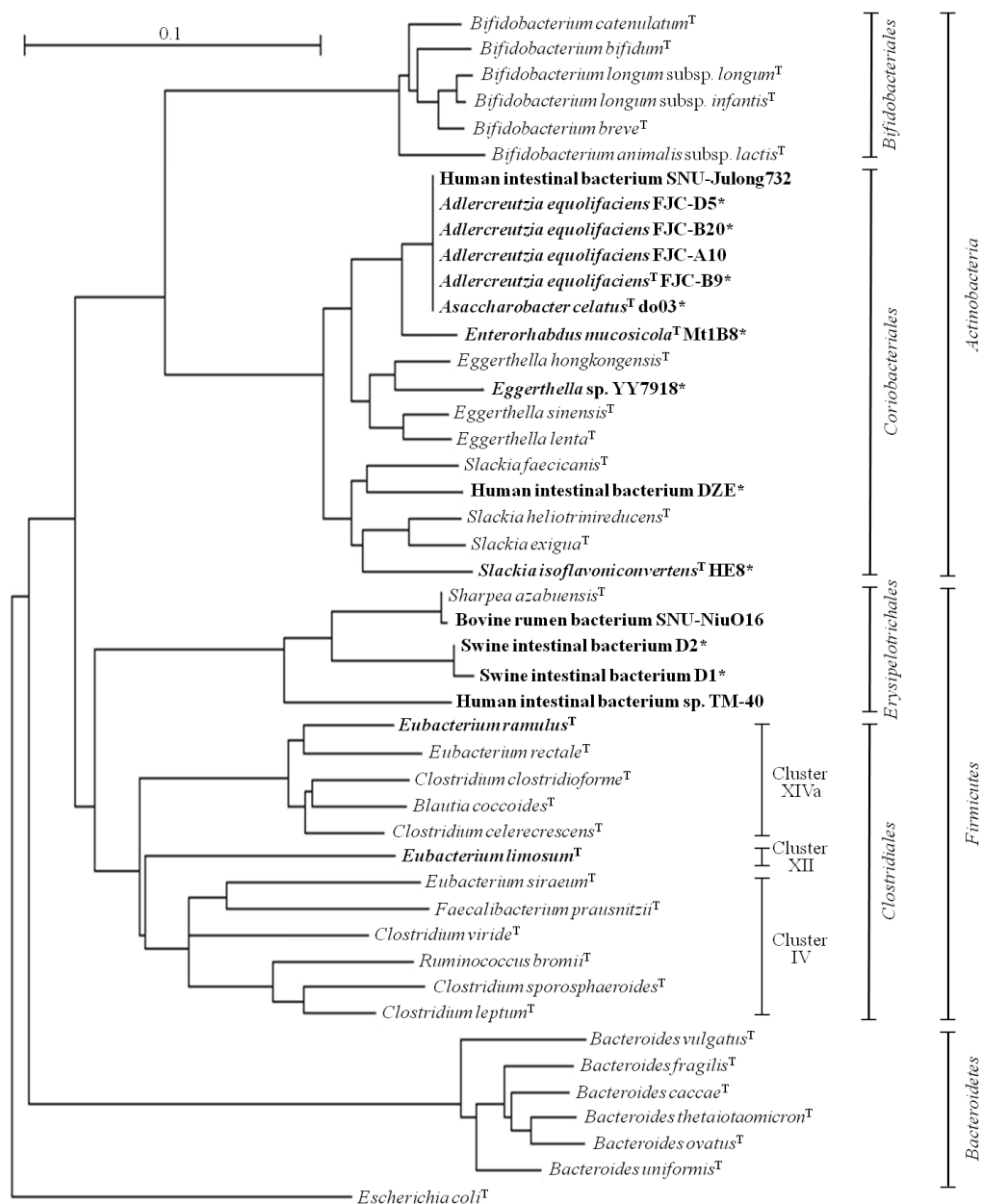


Figure 1. Phylogenetic tree showing representative species of the major taxa of the intestinal microbiota and, in bold, the bacteria which are known to be involved in isoflavones metabolism. The superscript * indicates bacteria which are capable of daidzein conversion into equol. The comparison is based on the 16S rRNA gene sequences, available from the Ribosomal Database Project. The superscript ^T indicates type strain bacteria.

The absorption efficiency of the conjugated forms is generally poor, causing the bioavailability of glucosides, acetylglucosides, and malonylglucosides to be low. Thus, activation of naturally occurring conjugated isoflavones, with the release of the bioactive aglycone, is necessary to promote the absorption and to enable further biotransformation by colonic microbiota.

Upon ingestion, small intestinal brush-border membrane enzymes and microbial β -glucosidases are involved in the removal of the glycosyl group. The hydrolysis releases the aglycones, which are readily absorbed and become bioactive. Glucosidases are common and widely distributed in nature. A major cytosolic β -glucosidase has been detected in a variety of mammalian tissues (McMahon et al. 1997). It consists of an enzyme with broad specificity β -glucosidase activity, capable of hydrolyzing a variety of aryl β -D-glycosides. However, the major contribution to activation of phytochemicals seems to be carried out by bacterial enzymes. Potentially, bacterial enzymes of the small intestine can hydrolyze all glycoconjugates. On the contrary, the enzymes of colonic bacteria exert their activity only on molecules which escape hydrolysis and absorption in the small intestine. In the colon, the microbiota produces a number of glycosyl hydrolases which act on a variety of glycosidic bonds, including those of the glycoconjugates of plant secondary metabolites.

A major part of gut anaerobic bacteria is charged with the task of breaking down undigested polysaccharides and fermenting the resulting monosaccharides to short-chain fatty acids (SCFA). To perform this metabolism, a vast number of enzymes such as β -glucuronidase, β -glucosidase, β -xylosidase, α -arabinosidase, β -galactosidase, is produced by intestinal bacteria (Scheline 1973). These enzymes can be located extracellularly, cell-bound or released into the lumen environment by cell lysis. Several studies investigated bulk enzyme activities in faecal samples. A high interindividual variation in enzymatic hydrolytic activities, including β -glucosidase, has been demonstrated in human faeces (McBain and Macfarlane 1998). These differences have been attributed to factors which are affected by the diet, such as the composition of the gut microbiota and the availability of carbon and energy sources. For instance, β -glucosidase activity increased in faeces following soy consumption by human volunteers (Weiserman et al. 2004).

In decreasing order of concentration, the bacteria belonging to the groups of *Bacteroidetes*, *Clostridium* cluster XIVa, *Clostridium* cluster IV, and *Actinobacteria* (mostly *Atopobium* and *Bifidobacterium*) dominate the colonic microbiota (Harmsen et al. 2002, Aminov et al., 2006, Mueller et al. 2006, Mariat et al. 2009). β -glucosidase activity has been found against the model substrate *p*-nitrophenyl β -D-glucopyranoside within members of all these major bacterial groups, mostly as a cell-bound activity (McBain and Macfarlane 1998, Nakamura et al. 2002, Dabek et al. 2008). Thus, it is conceivable that diverse bacteria may be involved in the hydrolysis of isoflavones glycoconjugates. Even though β -glucosidase seems to be widespread among the above bacterial groups, marked differences have been observed among the members of the same group.

Only few strains of *Bacteroides* have been screened for β -glucosidase and some of them exhibited high β -glucosidase activity, while other were negative to the assay. In a study, three strains of the genus *Bacteroides* (*B. ovatus*, *B. fragilis*, and *B. vulgatus*) were all positive for β -glucosidase, the highest activity being exhibited by *B. ovatus* (McBain and Macfarlane 1998). Diversely, β -glucosidase was not found in other two strains of *B. ovatus* and *B. vulgatus*, but only in one of *B. thetaiotaomicron* (Dabek et al. 2008). However, data concerning hydrolysis of glycosylated isoflavones by *Bacteroides* are lacking. It is possible that this bacterial group, which possess a major array of enzymes for sugar hydrolysis and can break down a large variety of glycosidic bonds, is involved in the release of isoflavone aglycones from glycoconjugates as well.

Within *Clostridiales*, β -glucosidase activity have been examined in 22 strains of cluster XIVa and 6 of cluster IV, including both human faecal isolates and reference strains (Dabek

et al. 2008). Among the strains of cluster XIVa, β -glucosidase was present in 8 strains of *Roseburia* (*R. intestinalis*, *R. fecis*, *R. hominis*, *R. inulinivorans*), 3 of *Eubacterium rectale*, 2 of *Coprococcus*, 1 of *Ruminococcus obeum*, and 1 of *Butyrivibrio fibrisolvens*, while it was not found in 2 strains of *Eubacterium hallii*, 2 of *Coprococcus comes*, 1 of *Anaerostipes caccae*, and 2 unidentified isolates. Among the strains of cluster IV, only 1 strains of *Eubacterium siraeum* and 1 of *Ruminococcus* sp. produced β -glucosidase, while 1 strain of *Ruminococcus bromii* and 3 of *Faecalibacterium prausnitzii* did not (Dabek et al. 2008).

Most information about β -glucosidase production by human intestinal bacteria is available for the genus *Bifidobacterium*, since considerable interest has focused on the utilization of bifidobacteria to hydrolyze isoflavone glycoconjugates in fermented soy products. Likewise the other saccharolytic intestinal bacteria, bifidobacteria can produce a number of diverse glycosyl hydrolases which are necessary to hydrolyze oligo and polysaccharide into fermentable carbohydrates in the colon (Schell et al 2002). Many strains belonging to diverse bifidobacterial species have been selected for their ability to hydrolyze the glycosylated forms of soy isoflavones (Bae et al. 2000, Bae et al. 2002, Tsangalis et al. 2002, Bae et al. 2004, Cheng et al. 2004, Marotti et al. 2007, Dabek et al. 2008, Raimondi et al. 2009,). Coherently, several strains of *Bifidobacterium* have been demonstrated to produce β -glucosidase (Marotti et al. 2007, Raimondi et al. 2009) and many of them exhibited an enzymatic activity that was higher or at least comparable than that of most strains of *Bacteroidetes* and *Clostridiales* (Dabek et al 2008). Among bifidobacteria, low production or lack of β -glucosidase was observed only within the species *B. longum* (Marotti et al. 2007, Raimondi et al. 2009). The absence of β -glucosidase activity in a few strains was in agreement with the incapability of hydrolyzing daidzin (Raimondi et al. 2009).

In conclusion, currently available information do not allow to establish which bacterial group is the major responsible for deconjugation of isoflavones *in vivo*. *Bacteroidetes* and *Clostridiales* are numerically predominant by approximately one magnitude order over bifidobacteria (Harmsen et al. 2002). Conversely, β -glucosidase seems to be more widespread within the genus *Bifidobacterium*, which have been also reported to produce higher activity albeit they are less numerous.

Bacterial Transformations of Isoflavone Aglycones

Isoflavone aglycones are extensively transformed by intestinal bacteria into a variety of metabolites which may exert enhanced or decreased biological activity (Aura 2008, Selma et al. 2009, Crozier et al. 2009). The production of different end-products by intestinal bacteria is affected by differences in the structure of the parent aglycone. In fact isoflavones differ in the number and/or position of hydroxyl and methoxyl substituents of A and B rings which may promote certain types of reactions (Figure 2).

The major transformations occurring to all classes of isoflavones generally start with the hydrogenation of the activated double bond between C-2 and C-3 of the C-ring. This reduction generates dihydroisoflavones, such as dihydrodaidzein, dihydrogenistein, and dihydroglycitein (Figure 2). The involvement of the gut microbiota in the production of all these dihydroisoflavones have been extensively documented and several isolates of intestinal

bacteria have been discovered to carry out this reaction (Hur et al. 2000, Wang et al. 2005a, Wang et al. 2005b, Tamura et al. 2007). Since germ-free rats excreted dihydrodaidzein and dihydrogenistein with urine, it is likely that the production of dihydroisoflavones can occur in the liver without any contribution of intestinal bacteria, presumably through a cytochrome P450 mediated reaction (Bowey et al. 2003). However, it is necessary that the hydrogenation is carried out by intestinal bacteria to make dihydroisoflavones available in the colon for further microbial transformation. Moreover, the hydrogenation of the double bond causes the appearance of a chiral center on C3 position of dihydroisoflavone, but a tautomerization reaction likely occurs to convert the R-dihydroisoflavone into the S-isomer (Wang et al. 2005b, Kim et al. 2009). Dihydroisoflavones constitute branch point of two divergent routes of bacterial transformation. They may undergo a reductive pathway yielding equol and equol-related compounds or, alternatively, the cleavage of C ring which, in certain cases, may lead to the fission of the molecules into two moieties (Figure 3, Figure 4, Figure 5).

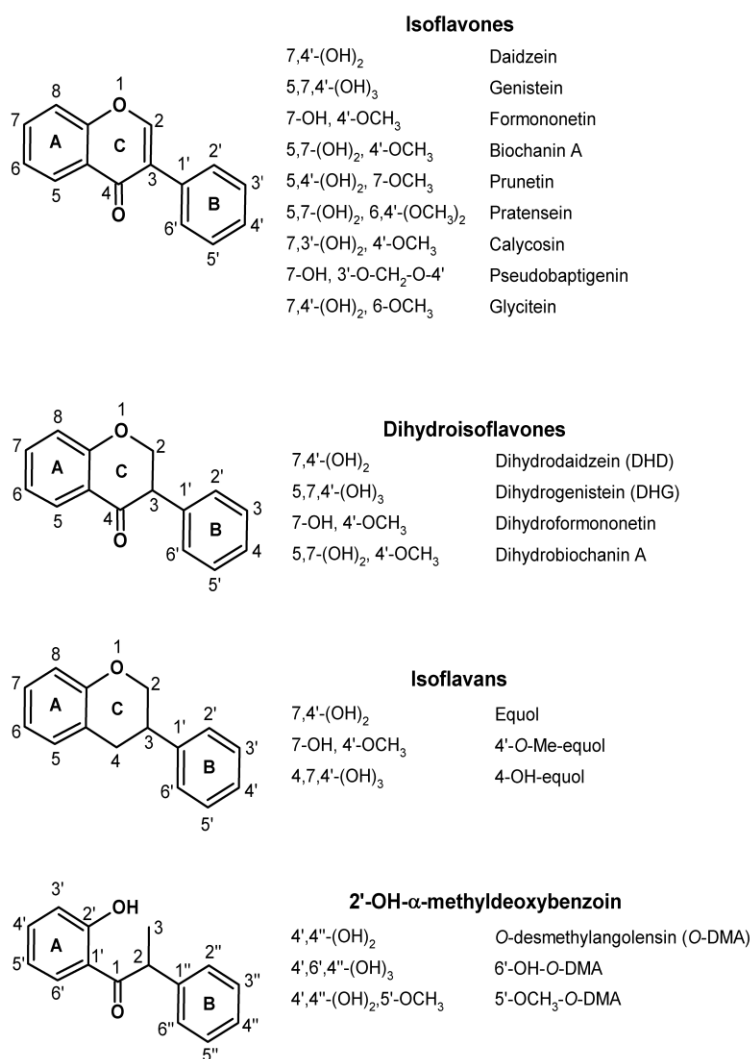


Figure 2. Chemical structures of the major dietary isoflavones and their bacterial metabolites

Production of equol-related compounds requires the reduction of the cheto-group in position 4 of the C-ring of isoflavones, yielding a compound related to 4-hydroxy-equol (Figure 3). This molecule possesses two chiral centers on positions C3 and C4, but only the *cis*-diastereoisomer of 4-hydroxy-equol, and specifically 3*S*, 4*S*-configuration, is selectively produced by the gut microbiota. (Setchell et al. 2005, Rüfer et al. 2006). The hydroxyl group on position C4 can be removed by intestinal bacteria, producing a reduced compound similar to equol. Therefore, S-equol is the sole enantiomer arising from daidzein (Setchell et al. 2005). The configuration of the chiral centers formed within the compounds which are produced from isoflavones other than daidzein (e.g. genistein or glycitein) has never been investigated, but it is conceivable that it is the same of daidzein metabolites.

The bacterial cleavage of C-ring of dihydroisoflavones always starts off with the break of the bond between the oxygen in the pyrone ring and the C-2 to yield a compound related to *O*-desmethylangolensin (*O*-DMA) (Figure 2). If this compound does not present a hydroxyl group on position 6' (corresponding to position 5 of the A-ring of the isoflavone), depending on structure of the parent aglycone, no further reactions occur on C-ring. Therefore C-ring cleavage of dihydrodaidzein does not progress beyond *O*-DMA.

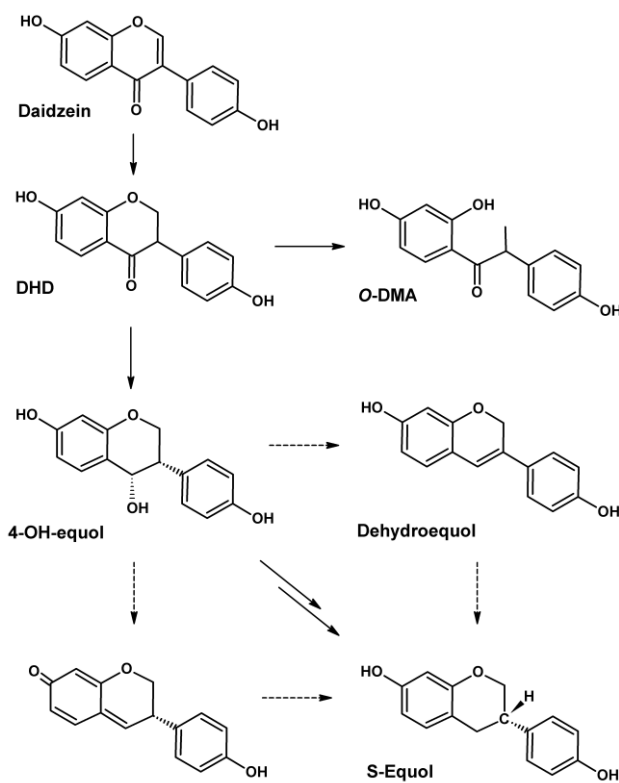


Figure 3. Intestinal bacterial metabolism of the isoflavone daidzein to *O*-DMA and equol. The dashed arrows indicate hypothesized pathways

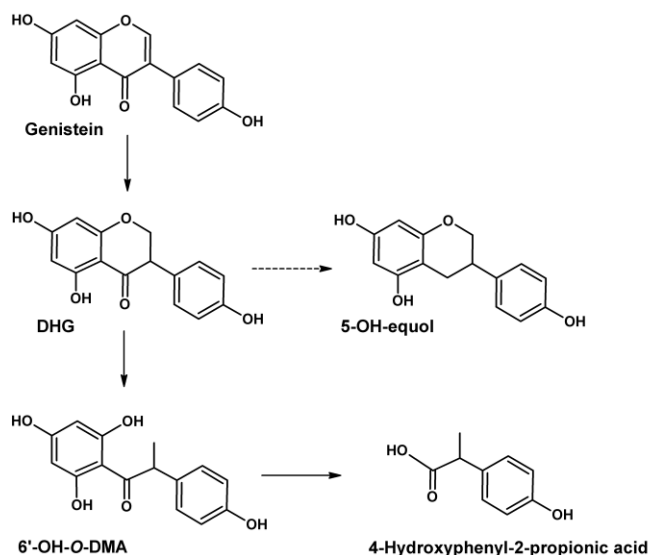


Figure 4. Intestinal bacterial metabolism of the isoflavone genistein: C-ring fission and production of 5-OH-equol. The dashed arrow indicates a reaction which is performed by intestinal isolates which have never been demonstrated *in vivo*.

On the contrary, if the compound related to *O*-DMA bears a hydroxyl group in position 6', the bond between position 1 and 1' is broken by intestinal bacteria and the molecule is cleaved into two moieties (Figure 4): phloroglucinol, originating from isoflavone A-ring, and a compound related to 2-(4-hydroxyphenyl)-propionic acid (HPPA), originating from B-ring. Similar ring cleavage occurs in the bacterial metabolism of other flavonoids, such as the flavanol quercetin, the flavone apigenin, the flavanone hesperetin, and the flavanols catechins. Likewise genistein, all these flavonoids possess a hydroxyl group in position 6' and can be similarly cleaved into phloroglucinol and a derivative of HPPA (Rechner et al. 2004). Furthermore, different schemes of ring cleavage occur in quercetin metabolism, giving rise to a variety of fission products (e.g. hydroxyphenylacetic and hydroxybenzoic acids), but they have never been reported for genistein and the other isoflavones (Rechner et al. 2004, Aura 2008, Selma et al. 2009).

Daidzein

Daidzein (4', 7-dihydroxyisoflavone) is the major isoflavone in soy germ (Figure 3). In soybeans and non-fermented food it almost totally occurs in the conjugated forms of glucoside (daidzin), acetylglucoside (acetyldaizidin) and malonylglucosides (malonyldaizidin). Only after the release of the sugar moiety by human and bacterial β -glucosidase, the aglycone can be absorbed through the gut epithelium or undergo a reductive metabolic pathway catalyzed by components of the gut microbiota. Among soy isoflavones, daidzein is the most studied because of the great biological activity of equol, one of its major metabolites. Equol exists in the two chiral forms R and S, where S-equol is the most effective in stimulating an estrogenic response among the isoflavone derivatives and has a longer half life and a superior antioxidant activity (Setchell and Cassidy 1999, Setchell and Cole 2003, Wang et al. 2007).

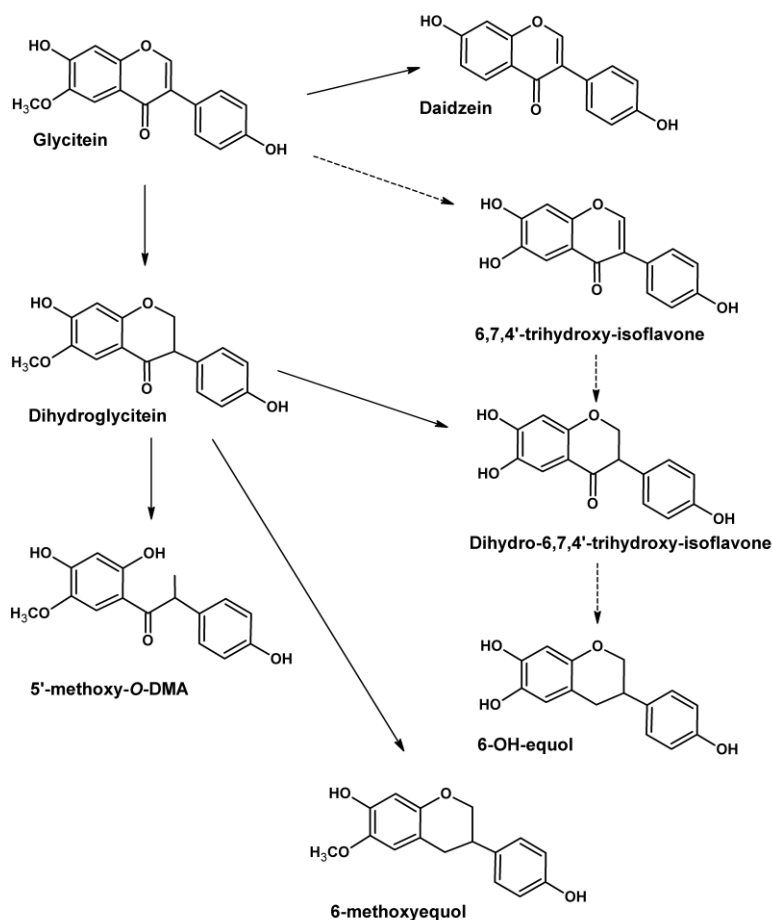


Figure 5. Intestinal bacterial metabolism of the isoflavone glycitein. Dashed arrow indicate hypothesized bacterial reactions.

The reductive pathway of daidzein starts with the production of dihydrodaidzein (DHD). DHD can undergo C-ring cleavage, leading to the production of *O*-desmethylangolensin (*O*-DMA) or, conversely, can be transformed into S-equol through reduction reactions (Atkinson et al. 2005, Wang et al. 2005, Wang et al. 2007, Yuan et al. 2007, Kim et al. 2009) (Figure 3).

The role of the gut microbiota in the reductive pathway of daidzein is well documented. Germ-free animals do not produce S-equol or *O*-DMA (Bowey et al. 2003), and *in vitro* incubation of human fecal samples with daidzein can give equol and/or *O*-DMA as products (Atkinson et al. 2004). Great interindividual variability affects the capacity to produce S-equol and *O*-DMA, as a consequence of the impact of the diverse composition of colonic microbiota (Zhang et al. 1999). In fact, the production of S-equol occurs only in 30-40% of people (Lampe et al. 1998), whereas approximately 80-90% can produce *O*-DMA, not all the S-equol producers being *O*-DMA producers and *vice versa*.

The central role of the gut microbiota in S-equol production was demonstrated through bioconversion experiments with fecal cultures. Fecal samples from equol producers successfully converted daidzein into S-equol, while fecal samples from non-producers did not, albeit some of the latter produced *O*-DMA and DHD.

Reduction to Dihydrodaidzein

The first step of daidzein metabolic pathway is the reduction to DHD, through the hydrogenation of the activated double bond between C-2 and C-3 of the C-ring (Figure 3). This step attracted great interest, since DHD represents the branch point of different fates of daidzein metabolism. DHD is a stable compound found in feces incubated with daidzein and in the urine of individuals consuming soy isoflavone, that can be easily extracted and revealed by HPLC coupled with UV (Chang and Nair 1995, Joannou et al. 1995).

Since the gut microbiota consists of a multitude of species, it is likely that several microbial groups can carry out the reduction of daidzein to DHD. Single strains performing this conversion have been isolated. Some of them can perform only this reaction of daidzein metabolism, such as the *Clostridium*-like strains SNU Niu-O16, TM-40, and HGH6, still lacking a final taxonomic classification, but exhibiting similarity to members of *Erysipelotrichales* (Figure 1). The strain Niu-O16 was isolated from bovine rumen (Wang et al. 2005a), whereas the strains TM-40 and HGH6 were obtained from human feces (Hur et al. 2000, Tamura et al. 2007). Transformation of daidzein by Niu-O16 resulted in (*R*)- and (*S*)-DHD enantiomers with the same abundance, but it was not determined whether this was due to a non-enantioselective reduction or to a tautomerization reaction (Wang et al. 2005b).

O-DMA Production and Isoflavone Degradation

The intestinal microbiota acts by two distinctive pathways to convert daidzein to the more estrogenic metabolite equol or to the less estrogenic one *O*-desmethylangolensin (*O*-DMA) (Joannou et al. 1995) (Figure 3). In this latter case daidzein is first converted to dihydrodaidzein, then to *O*-DMA through 2-dehydro-*O*-DMA (Hur et al. 2002). Among several intermediates, *O*-DMA and S-equol were the first to be isolated and characterized, since they are easily extracted and detected. They occur in urine and blood of humans and animals and were found as final products of *in vitro* experiments with fecal cultures as well (Chang and Nair 1995, Joannou et al. 1995, Heinonen et al. 1999).

The C-ring cleavage of daidzein can be accomplished by the colonic species *Eubacterium ramulus* (Schoefer et al. 2002), the major product observed being *O*-DMA. *E. ramulus* is a member of *Clostridium* cluster XIVa and is regarded as one of the major isoflavones degrading bacteria in the human gastrointestinal tract (Schoefer et al. 2002) (Figure 1). Consistently, during a flavonoid-free diet, both total cell counts of intestinal bacteria and *E. ramulus* decreased significantly, while there was an increase in the *E. ramulus* amount of up to 10-fold during a flavonoid-rich feeding period (Schoefer et al. 2002). These results suggest that dietary secondary plant metabolites may influence the composition of intestinal microbiota (Blaut et al. 2003).

Among intestinal bacteria, also *Clostridium* sp. HGH 136, isolated from human feces, cleaved the C-ring of daidzein to *O*-DMA under anoxic conditions. Dihydrodaidzein and 2-dehydro-*O*-DMA were transient intermediates and did not accumulate in the culture (Hur et al. 2002). Also the human strain SY8519, belonging to a novel genus in the *Clostridium* cluster XIVa (Yokoyama et al. 2009), produced *O*-DMA from daidzein. Being phylogenetically related to *E. ramulus*, it is possible that it can metabolize a broad spectrum of polyphenols (Yokoyama et al. 2009).

Equol Production

Extensive search for intestinal bacteria capable of producing equol has been carried out and most reactions of the biotransformation have been unraveled. DHD is transformed by components of fecal microbiota into S-equol through *cis*-4-hydroxy-equol, the major diastereoisomer of 4-hydroxy-equol occurring in urine samples and in fecal cultures (Heinonen et al. 1999, Setchell et al. 2005, Rüfer et al. 2006). 4-hydroxy-equol is converted to equol by either reductive rearrangement or by dehydration and subsequent reduction (Joannou et al. 1995, Kim et al. 2009) (Figure 3).

Several fecal cultures and consortia of intestinal bacteria convert daidzein into equol (Chang and Nair 1995, Hur et al. 2000, Rafii et al. 2007) (Figure 1). A stable mixed culture transforming daidzein into equol was obtained from a fecal sample of an equol-producing human subject. It was composed of *Lactobacillus mucosae*, *Enterococcus faecium*, *Fingoldia magna* and *Veillonella* sp. The addition of this bacterial consortium to a fecal culture that could not metabolize daidzein provided the capacity to produce equol (Decroos et al. 2005). A diverse mixed culture, containing the *Clostridium*-like strain SNU Niu-O16 and *Eggerthella* sp. Julong 732, was able to produce S-equol (Wang et al. 2007). The former strain was capable to reduce daidzein to dihydrodaidzein, the latter produced S-equol, and together they completely and stereoselectively (100% e.e.) converted daidzein into S-equol. The strain named SNU Niu-O16 yielded a racemic mixture of R and S dihydrodaidzein, while *Eggerthella* sp. Julong 732 did not exhibit racemase activity. Thus, it is conceivable that the complete and stereoselective bioconversion resulted from enantioselective preference of *Eggerthella* sp. Julong 732 toward S-dihydrodaidzein, with a putative tautomerization reaction that converted R-dihydrodaidzein into the S-isomer (Wang et al. 2005a, Wang et al. 2005b, Wang et al. 2007, Kim et al. 2009).

Considerable efforts focused on the search for single bacterial strains capable to carry out the complete bioconversion have been unsuccessful for a long time. The general approach was to screen bacteria isolated from feces of equol-producing subjects. Most of research explored the ability of commensal lactobacilli and bifidobacteria to carry out the complete reaction, in the perspective to use positive strains as probiotic supplements or starters for the production of equol-enriched soy-based fermented food. These studies failed to identify positive strains and demonstrated that few lactobacilli strains, for instance *L. mucosae*, can participate to some steps of equol production (Decroos et al. 2004, Wang et al. 2005b). Up to now, the involvement of bifidobacteria in the reduction of daidzein toward S-equol seems to be excluded, even if they exert an important role in the hydrolysis of isoflavone glycoconjugates to release the aglycone. Albeit bifidobacteria have never been reported among the single bacteria which perform the whole reductive bioconversion from daidzein to S-equol (Wang et al. 2005a, Minamida et al. 2006, Tamura et al. 2007, Wang et al. 2007, Minamida et al. 2008, Maruo et al. 2008, Yu et al. 2008, Matthies et al. 2008), one study described the formation of S-equol in soymilk fermented with some strains of *Bifidobacterium* (Tsangalis et al. 2002). Nonetheless, S-equol has never been claimed again as a bioconversion product of soymilk isoflavones by bifidobacteria and, in a further investigation, 22 bifidobacteria failed to transform daidzein into reduced metabolites under all the experimental conditions, excluding any role in the reductive pathway of daidzein toward the production of S-equol (Raimondi et al. 2009).

Some intestinal strains which carry out the bioconversion of daidzein to S-equol self-sufficiently have been recently isolated and characterized from the feces of humans and animals. In some cases, the phenotypic and biochemical features and the phylogenetic position of the new isolates impelled the proposal of new bacterial genera and/or species or caused the description of previously defined genera and/or species to be updated (Minamida et al. 2006, Maruo et al. 2008, Matthies et al. 2009). Since identification of an increasing number of equol producing bacteria is in progress, and many of them are the forerunner of novel species, the list of equol-producing bacteria is likely far to be conclusive.

Nearly all the equol-producing bacterial isolates have been classified within the high G+C% content gram-positives, in the class of *Actinobacteria*, and specifically in the family of *Coriobacteriaceae*, whose role in isoflavonoid metabolism within the mammalian intestine seems crucial. Within *Coriobacteriaceae*, equol-producers belong to the genus *Eggerthella* and *Slackia* (Jin et al. 2008, Yokoyama et al. 2008, Matthies et al. 2009), and to the species *Asaccharobacter celatus* and *Adlercreutzia equolifaciens* (Minamida et al. 2006, Minamida et al. 2008, Maruo et al. 2008, Matthies et al. 2008) (Figure 1).

The human strain *Eggerthella* sp. YY7918 converts daidzein into DHD and completes the transformation to S-equol with high stereoselectivity. Unlike other *Eggerthella* strains, it can't hydrolyze daidzin, due to the lack of β -glucosidase activity. Furthermore, it doesn't carry out any transformation of other isoflavone aglycones, such as glycitein, genistein, and formonetin (Yokoyama et al. 2008). Few strains within the family of *Coriobacteriaceae* metabolize only DHD to equol, but other strains do not present equol-producing activity (Minamida et al. 2006, Minamida et al. 2008, Maruo et al. 2008). Therefore, a reasonably wide diversity in equol-producing capability seems to exist within this family. Furthermore, whereas growing cells of human strain *Slackia isoflavoniconvertens* HE8 produced equol in high amount (62% of yield), they hardly converted daidzein when it was added during the stationary growth phase, suggesting that isoflavone conversion might be inducible (Matthies et al. 2009).

Few equol-producing intestinal isolates were not classified within the family of *Coriobacteriaceae*, such as *Eubacterium* sp. (Yu et al. 2008). Furthermore, three strains of *Bacteroides ovatus*, *Streptococcus intermedius*, and *Streptococcus constellatus* were patented for equol production (Uchiyama et al. 1999). However, the ability of *B. ovatus*, *S. intermedius*, and *S. constellatus* seems to be questionable, since these species have never been claimed again as equol producers since the patent registration.

It is important to mention also the strain *Lactococcus garviae* 20-92 that, albeit isolated from human fecal samples (Uchiyama et al. 2007), is typically found in fish and aquacultures and does not normally occur in the human gut microbiota, although it is not pathogenic or toxic. This strain converts daidzein to equol during the stationary phase of growth, and represents the first lactic acid bacterium which perform the complete transformation of daidzein into equol (Uchiyama et al. 2005, Yee et al. 2008, Setchell et al. 2009).

Effects of the Gut Environment on Equol Production

The pivotal role of the gut microbiota in daidzein metabolism and equol production is unquestionable. Therefore, it seems possible to improve equol production through changes in the environmental conditions in the hindgut, hence modulating the microbiota. In particular, the diet and other physiological factors (e.g. the intestinal transit time and redox potential),

influence the microbiota composition and/or activity and affect equol production (Setchell et al. 1984).

Several studies established a comparison between the habitual diet of equol-producers and non-producers, but led to contrasting conclusions. A positive relationship between carbohydrate and fiber intake and equol production was identified in people consuming a western diet (Lampe et al. 1998, Rowland et al. 2000). Furthermore it was reported that subjects non-producers were converted to equol-producers with continual soy ingestion (Lu et al. 1996). However, it is hard to be conclusive about this argument and it seems that the question is still open, since a diverse study demonstrated that it was impossible to induce equol production in non-producers by supplementing diets with high-fiber wheat bran cereal or soy protein (Lampe et al. 2001).

Equol production by single strains or mixed microbial culture was affected, *in vitro*, by environmental conditions, such as the presence of hydrogen and the addition of FOS, arginine, butyric and propionic acids (Decroos et al. 2005, Minamida et al. 2006). Hydrogen gas and SCFA occur within the gut, since they are fermentation products of diverse intestinal bacteria. H₂ exerted a major impact on daidzein metabolism by fecal cultures: incubation with H₂ increased equol production, while equol was not detected in presence of N₂ or metallic palladium. Furthermore, addition of SCFA influenced equol production *in vitro*: butyric and propionic acids enhanced equol production, while the opposite effect was attained by acetic acid (Decroos et al. 2005, Minamida et al. 2006). It is known that the production of SCFA and of H₂ are connected through complex syntrophic relationship among bacteria, involving some of the most represented bacterial groups within the gut microbiota. In fact, as a result of the mutualistic cross-feeding among bacteria, hydrogen production and consumption, acetate production and consumption, and production of butyrate and propionate are strictly related each other (Duncan et al. 2002; Flint et al. 2007). Thus, it was proposed that the influence of SCFA on equol production is somehow in relationship to bacterial H₂ consumption and production (Duncan et al. 2002, Yu et al. 2008).

The investigation of whether the prebiotic fructo-oligosaccharides (FOS) may improve equol production lead to contrasting conclusions. The addition of FOS to a fecal microbial culture inhibited equol production (Decroos et al. 2005), while rats fed a diet supplemented with FOS exhibited increased intestinal bioavailability of daidzein (Uehara et al. 2001). Since FOS fermentation increases hydrogen release by certain intestinal bacteria (Cummings et al. 2001), it was proposed that FOS may stimulate equol production (Decroos et al. 2005). However, FOS are known to modify the composition of the human colon microbiota, causing a significant improvement of the population of bifidobacteria (Gibson and Roberfroid 1995, Hopkins and Macfarlane 2003), a genus which has a major role in hydrolysis of isoflavone glycoconjugates, but which is incapable of daidzein metabolism.

Genistein

Genistein (4', 5, 7-trihydroxyisoflavone) is the second soy isoflavone for importance and occurrence after daidzein (Dixon et al. 2002) (Figure 2). It is found in soybean in high amounts (up to 1 g Kg⁻¹) mostly in conjugated form within the cotyledons (Franke et al. 1995). Pharmacological effects of genistein include tyrosine kinase inhibition,

chemoprotectant activities against cancer, and cardiovascular disease (Banerjee et al. 2008), but few information are still available about biological activity of its metabolites.

Likewise daidzein, genistein undergoes extensive biotransformation by the gut microbiota (Figure 4). It is first converted into dihydrogenistein (DHG) through the reduction of the activated double bond between C-2 and C-3 to a single bond. This reaction is the same occurring to daidzein and the above mentioned strains SNU sp. Niu-O16 and HGH6, converting daidzein to DHD, were capable of reducing genistein to DHG as well, but could not carry out any further transformation of both DHD and DHG (Hur et al. 2000, Wang et al. 2005b). Likewise (*R*)- and (*S*)-DHD, (*R*)- and (*S*)-DHG were produced by the strain SNU Niu-O16 with the same abundance, probably as a consequence of the tautomerization reaction (Wang et al. 2005b).

As well DHD, DHG may be subject to two different fates, such as the cleavage of C-ring or the transformation into 5-OH-equol through a reductive pathway similar to the one leading to equol. For a long time, it has been assumed that the equol-like transformation of DHG could not occur, because the putative intermediate (tetrahydrogenistein) and end-product (5-OH-equol) have never been observed in urine or feces nor identified when genistein or DHG were incubated with equol-producing fecal cultures (Griffiths and Smith 1972, Chang and Nair 1995). Albeit 5-OH-equol has been never found in urine, feces, or blood, and it is still unclear whether it is produced in the hindgut (Heinonen et al. 1999, Coldham et al. 1999, Coldham et al. 2002), few intestinal bacterial strains capable to transform genistein into 5-OH-equol have been identified among the ones which can transform daidzein into equol (Jin et al. 2008, Matthies et al. 2008, Matthies et al. 2009).

Genistein metabolites arising from the cleavage of C-ring, such as 4-ethylphenol, have been identified in fecal cultures and in animal studies (Griffiths and Smith 1972, Chang and Nair 1995). Other metabolites of genistein, such as dihydrogenistein (DHG) and 6'-hydroxyl-*O*-DMA (6'-OH-*O*-DMA), were found in human urine (Kelly et al. 1993, Joannou et al. 1995, Heinonen et al. 1999). Furthermore, a human intestinal strain of *Eubacterium ramulus* cleaving the C- ring and degrading genistein has been identified (Schoefer et al. 2002). This strain cleaved the C-ring of genistein producing 6'-OH-*O*-DMA, which was further cleaved into two moieties, phloroglucinol and 2-(4-hydroxyphenyl)-propionic acid (HPPA), originating from A-ring and B-ring, respectively. Phloroglucinol is utilized as a substrate by intestinal bacteria, thus it is quickly degraded and could therefore not be detected (Schoefer et al. 2002, Rechner et al. 2004). Any further conversion of HPPA has never been observed by means of human intestinal bacteria (Joannou et al. 1995), even if it is transformed into 4-ethylphenol in some animal models (Batterham et al. 1965, Chang and Nair 1995).

The microbial transformation of genistein into HPPA and 4-ethylphenol represents a substantial loss of biological activity, since estrogenic or other healthy properties have never been reported for these compounds (Steer et al. 2003). Therefore, the prevention of C-ring cleavage is of interest to maintain the biological activity of genistein. Albeit prediction of *in vivo* behaviors based on results from *in vitro* experiments should be carried out with caution, the addition of FOS to fecal cultures preserved genistein from degradation. It was proposed that FOS limited degradation of genistein through the selective stimulation of bifidobacteria. In fact they cannot perform reduction of complex molecules (McMullen et al. 2006, Chun et al. 2007, Raimondi et al. 2009, Iqbal and Zhu 2009) and their growth can hinder the growth of other bacteria responsible for genistein metabolism (Steer 2003).

Other Isoflavone Compounds

Other naturally occurring isoflavones include glycitein, formononetin, biochanin A, prunetin, pratensein, calycosin, and pseudobaptigenin (Figure 2). They differ in the number and in the presence of hydroxy and methoxy groups. Because of the limited occurrence in the human diet, their metabolism has not been studied in detail. Only some information about glycitein, formononetin, biochanin A and puerarin are present.

Glycitein

Glycitein (7, 4'-dihydroxy-6-methoxyisoflavone) (Figure 2) accounts for less than 10% of the total isoflavones in soybeans and soybean foods and for about 50% in soy germ (Song et al. 1998). A mouse uterine growth assay demonstrated that glycitein is more estrogenic than genistein, (Song et al. 1999), but, probably because of its scarcity in soy-based foods, its metabolism has not been explored yet.

Glycitein is structurally similar to daidzein, with the exception of the methoxyl group at the 6-position. Diverse molecules arising from glycitein reduction, including dihydroglycitein, dihydro-6, 7, 4'-trihydroxyisoflavone, 5'-O-methoxyl-*O*-desmethylangolensin, and 6-O-methoxyl-equol (Figure 2), were found in the urine of soy consuming humans (Heinonen et al. 2003), likely as a consequence of gut microbiota transformation. In fact, the same metabolites were produced when glycitein was incubated in fecal cultures from the metabolite-producing subjects (Simons et al. 2005). These observations suggest that the first step in glycitein metabolism is the reduction to dihydroglycitein. Then, dihydroglycitein can undergo to three different fates. It can be *O*-demethylated to dihydro-6, 7, 4'-trihydroxyisoflavone, the major metabolite occurring in fecal cultures, which may be reduced to 6-hydroxyequol or cleaved to 5'-hydroxy-*O*-desmethylangolensin. Diversely, it can be subject to C-ring cleavage or to reductive transformations, yielding 5'-O-methoxyl-*O*-desmethylangolensin and 6-methoxyequol, respectively, two metabolites occurring in minor amounts in fecal cultures (Simons et al. 2005) (Figure 5).

Minor transformation pathways of glycitein include demethylation and demethoxylation. Some intestinal bacteria, such as *Eubacterium limosum* of *Clostridium* cluster XII (Figure 1), can demethylate glycitein *in vitro* to 6, 7, 4'-trihydroxyisoflavone (Hur and Rafii 2000), but this metabolite has never been detected in human urine. It was proposed that it may be reduced to dihydro-6, 7, 4'-trihydroxyisoflavone, although this reaction has not been demonstrated to occur in fecal cultures (Simons et al. 2005). The direct demethoxylation of glycitein at the 6-position results in daidzein, but this reaction does not occur commonly *in vivo* and is rare in human fecal cultures (Simons et al. 2005, Setchell et al. 2001) (Figure 5).

Red Clover Isoflavones: Formononetin and Biochanin A

The main isoflavones of red clover (*Trifolium pratense*) are formononetin, biochanin A (Figure 2), and their glycosidic conjugates, ononin, and sissotrin, respectively (He et al. 1996, Kleydus et al. 2001). Since estrogenic potency and antioxidant activity of isoflavones is significantly affected by the position and number of hydroxyl groups, biochanin A and

formononetin are less active than genistein and daidzein (Arora et al. 1998, Kuiper et al. 1998).

Due to the limited occurrence in the human diet, the metabolism of red clover isoflavones has not been studied in great detail. It is known that formononetin and biochanin A are readily demethylated by the intestinal microbiota to daidzein and genistein, respectively (Batterham et al. 1971). Then, daidzein and genistein go through their respective metabolic pathways. The extent of demethylation of the methoxy substituents of isoflavones depends on its position in the isoflavone structure: the demethylation of the methoxy group occurs more easily in ring B than in ring A (Hur et al. 2000). Similar observations were made for flavonoids as well, where demethylation occurred more easily at C6 than at C4' (Nielsen et al. 2000). However, minor amounts of reduced metabolites of formononetin and biochanin A, still bearing the methoxy group at the 4'-position, were identified in urine at low levels, suggesting that demethylation of the methoxy group at the 4'-position is the major transformation, which occurs almost quantitatively (Heinonen et al. 2004a, Heinonen et al. 2004b).

Puerarin

Puerarin is an isoflavone *C*-glucoside (daidzein-8-*C*-glucoside) occurring abundantly in the roots of the legume genus *Pueraria* (e.g. *P. thunbergiana* and *P. lobata* or Kudzu). Although puerarin does not occur commonly in food, especially in western diet, it is of great interest for herbal medicine, due to its potent biological activity against cardiovascular diseases (Tan et al. 2008). Certain human intestinal isolates can remove the *C*-glucosyl with high substrate specificity through reductive cleavage (Kim et al. 1998, Park et al. 2006). Hence, puerarin is transformed into daidzein, which may further be converted into equol (Jin et al. 2008).

Conclusion

Likewise hundreds of non-nutritional phytochemicals occurring in diet, isoflavones undergo several transformations upon ingestion by the commensal microbiota harbored within the gut. Bacterial transformations may greatly increase or compromise the biological activity of dietary isoflavones, thus they have been subject of considerable interest, particularly those yielding valuable products with superior biological activity, such as equol. This chapter aimed to summarize what is known about the bacterial metabolism of dietary isoflavones. Although it is being increasingly unraveled for few bacterial isolates, an exhaustive knowledge of this topic is not available so far, due to the complexity of the intestinal bacterial ecosystem. In fact, this bacterial community is composed of hundreds of bacterial species which may encode approximately 100 times as many genes as the human genome and may carry out a number of biochemical metabolic reactions with complex reciprocal synergies. The task of disclosing all the interactions occurring *in vivo* among the diverse bacterial species and dietary isoflavones is daunting. To address this issue, it is necessary a coordinated work in the genetics, physiology, and metabolism of intestinal bacteria and in enzymology and chemistry as well. Recent advances in omic approaches,

allowing the simultaneous analysis of huge numbers of genes and proteins, are now available to achieve a more comprehensive knowledge of the pathways occurring in isoflavones metabolism.

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