

# Equol: History, Chemistry, and Formation<sup>1,2</sup>

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## Abstract

Equol, first isolated from equine urine in 1932 and identified 50 years later in human urine as a metabolite of the soy isoflavones, daidzin and daidzein, is produced by intestinal bacteria in some, but not all, adults. This observation led to the term equol-producers to define those adults that could make equol in response to consuming soy isoflavones and the hypothesis that the health benefits of soy-based diets may be greater in equol-producers than in equol nonproducers. By virtue of a chiral center, equol occurs as a diastereoisomer and intestinal bacteria are enantiospecific in synthesizing exclusively the *S*-(-)equol enantiomer, an enantiomer that has selective affinity for the estrogen receptor- $\beta$ . Both enantiomers are of interest from a clinical and pharmacological perspective and are currently being developed as nutraceutical and pharmacological agents. The wide range of biological activities these enantiomers possess warrants their investigation for the treatment of a number of hormone-related conditions involving estrogen-dependent and androgen-related conditions. The following review describes the history, chemistry, and factors governing the intestinal bacterial formation of equol. *J. Nutr.* 140: 1355S–1362S, 2010.

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## Introduction

It is now 57 years since the first report appeared describing a new phenolic compound in an estrogenic fraction of pregnant mare urine (1). It was suggested that the compound be given the name equol, after the equine source of the material. Efforts to obtain large-scale quantities of the compound led to the recognition that it was also present in appreciable amounts in the urine of stallions and nonpregnant mares and the conclusion that it was not associated with the presence of high estrogen states. During

the autumn months, the amounts of equol declined and by winter it was impossible to isolate it from urine. The authors concluded that, "so far as can be determined, no dietary factor was the cause of this (seasonal) variation..." (2). It later became apparent that this was not the case when in SW Australia reports emerged of a catastrophic "failure to breed" associated with uterine abnormalities and endometriosis in sheep grazing on *Trifolium subterraneum* clover (3). Reductions in sperm counts and motility were also documented in ewes (4). This clover disease, as it was so-called, was found to be the result of extremely high circulating concentrations of equol, formed by ruminal bacteria from the ingestion of large amounts of the methoxylated isoflavone, formononetin, abundant in several indigent species of clover (5–7). Equol was even found as a component in urinary calculi of sheep and cattle (8). Equol has since been reported to be present in the urine and/or plasma of many other animal species, including cows (9), hens (10–14), monkeys (15,16), chimpanzees (17,18), dogs (19), mice (20), rats (20–22), and pigs (16,23), but there are marked differences in the extent of metabolism of isoflavones into equol by these species. Rodents, e.g., very efficiently convert daidzin/daidzein to equol (24), whereas pigs and humans have been reported to do this less efficiently (16,24). In the decades leading to 1970, a great deal of work was performed defining the metabolism of isoflavones and biological actions of equol (6,25–28). While its estrogenic effects were well documented based upon field observations and classical bioassays, it was not until after the discovery of the first estrogen receptor (ER)<sup>5</sup> in the mid-1960s (29) that the relative affinity of equol for the ER could be

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<sup>5</sup> Abbreviations used: BBM, brush border membrane; ER, estrogen receptor; ISP, isolated soy proteins; RMB, relative molar binding.

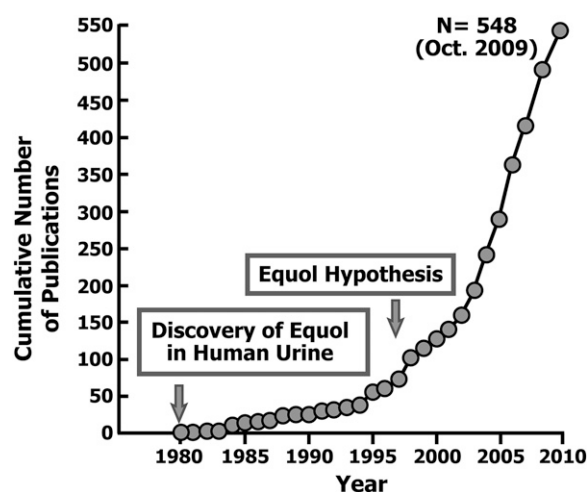
quantified. When the relative molar binding affinities of a number of phytoestrogens for sheep uterine ER were compared, equol was found to have much higher affinity than its precursor daidzein in competing with radioactive estradiol for binding to the cytosolic receptor (27), supporting the theory that it may be advantageous to be able to convert daidzein to equol (24).

There was little interest in equol for several decades until the chance discovery in 1980 of high concentrations of an unknown estrogen-like compound in rat urine (30) accompanying the mammalian lignans, enterolactone and enterodiols (31–33). At the time, it was referred to as compound 386/192, a notation for the molecular ion and base peak in the mass spectrum of its trimethylsilyl ether derivative. In common with most endogenous steroid hormones, including estrogens, it was conjugated predominantly to glucuronic acid and a lesser extent to sulfuric acid (34). Its presence in such high concentrations in rat urine fortuitously afforded a means of isolating sufficient quantities for structural elucidation studies by infrared spectroscopy, NMR, and GC-MS (35) and the subsequent confirmation that it was identical in chemical structure to the equol first isolated from pregnant mares urine in 1932 by Marrian et al. (1,2). This confirmation was made possible because one of us (K.D.R.S.) was gifted from the curator of the UK Medical Research Council's Steroid Reference Collection (the late Professor D.N. Kirk) the original 4.0 mg sample of equol isolated from pregnant mares urine by Marrian et al in 1932. Equol was also found to occur as a minor constituent in the urine of many adults. The link between equol and soy came about after a series of studies in which different plant-based foods were fed to rats maintained on a purified diet. The introduction of soy protein led to a huge increase in the urinary excretion of equol and following this observation, the soy isoflavone daidzin was isolated and shown to be a precursor to equol (35). It was also found that the introduction of soy protein to the diet led to an increased excretion of equol in some but not all adults (36), whereas *in vitro* incubation of cultured fecal flora from equol-producing individuals with either daidzein or soy protein resulted in the formation of equol (36). The finding of high concentrations of equol in the urine of adults consuming soy foods prompted the hypothesis that this nonsteroidal estrogen may be beneficial in the prevention and treatment of many hormone-dependent conditions (36).

Progress in research studies of equol was hampered by the lack of sufficient amounts of the compound for biological and clinical testing and by the divergent interest and focus on genistein, the other soy-derived isoflavone that was shown to be a potent inhibitor of tyrosine protein kinases (37) and a compound that was readily available in bulk. Almost 20 y after the finding of equol in human urine, it was proposed that the efficiency with which adults convert daidzein to equol when consuming diets containing soy foods could enhance the clinical effectiveness of soy-based diets—the so called equol-hypothesis (24)—and this has driven a resurgence of interest in equol, as is evident from the almost exponential increase since 1980 in the number of publications cited by a PUBMED search of this as a keyword (Fig. 1). At the recent 8th International Soy Symposium held in Tokyo, Japan, for the first time an entire session was devoted to equol (38). This overview will focus on some of the key areas related to this unique molecule and is not intended to be a comprehensive review of the topic.

## Chemistry

Equol [7-hydroxy-3-(4'-hydroxyphenyl)-chroman], an isoflavan, belongs to the general class of compounds referred to as



**FIGURE 1** Cumulative number of publications on equol by year since its first identification in human urine.

nonsteroidal estrogens. It has a molecular composition of  $C_{15}H_{14}O_3$  and a molecular weight of 242.27 Daltons. The heterocyclic structure contains 2 reactive hydroxyls and 1 relatively inert and unreactive oxygen in the central furan ring. Physicochemically, it is nonpolar and relatively insoluble in solution, something that should be considered when conducting *in vitro* experiments, particularly at high concentrations. It is also extremely acid-labile and can readily be destroyed (>60%) in the general work-up of samples, particularly if acidic hydrolytic steps are used (39). Despite having 2 phenolic rings, it exhibits poor UV absorption characteristics, meaning that HPLC with UV detection is unsuitable for its measurement in most biological fluids. As a result of a chiral carbon at position C-3 of the molecule, equol exists in 2 enantiomeric forms, *R*-(+) equol and *S*-(-)equol, and the latter is the natural diastereoisomer produced by intestinal bacteria in the intestine of humans and rats (40). This makes it distinct from its precursor isoflavone, daidzein, and the 2 other major isoflavones of soy, genistein and glycitein (41). Equol can be readily synthesized from daidzein by catalytic hydrogenation, but this yields the ( $\pm$ ) equol form (42) and it is the form that has been commercially available and mostly utilized in studies of its biological potency and properties. Indeed, unless otherwise stated, it can be assumed that all previously reported experiments used ( $\pm$ )equol and not the individual enantiomers. It cannot always be assumed that the racemate will behave in an identical manner to that of the individual enantiomers and this was recently shown to be the case for its pharmacokinetics (43). It is probable that biological effects may be underestimated when testing with the racemate and this may particularly hold true for binding affinities to receptors. The racemic mixture can be readily separated by chiral chromatography and the earliest studies used this approach to isolate sufficient amounts of each enantiomer to determine the estrogen binding affinities (40,44) and the pharmacokinetics (43). More recently, methods for the selective synthesis of *S*-(-)equol (45,46) and *R*-(+)equol (45) have been described. Methods for the synthesis of [ $^{13}C$ ]labeled isoflavone analogs (47–50) have been described that can represent suitable starting points for the preparation of stable-labeled [ $^{13}C$ ]equol for use as tracers in metabolic studies or for internal standards in stable-isotope dilution mass spectrometric assays (43). The synthesis of *S*-(-)equol and *R*-(+)equol by chiral chemistry (45) now affords the large-scale production of enantiomeric pure compounds for use in clinical and animal studies. Finally, the

production of *S*-(-)equol from daidzein-rich soy germ by a specific equol-producing bacterium, *Lactococcus garvieae* (51), offers an alternative biological means to producing specifically *S*-(-)equol (52). With these breakthroughs, it is now possible to study in some detail the effects of equol in animals and humans and such studies will likely shed further light on the relevance of this metabolite to the clinical outcomes.

### Role of intestinal bacteria in the formation of *S*-(-)Equol

The early evidence in support an intestinal bacterial origin for equol in humans and animals was as follows: 1) germ-free animals fed a soy diet do not excrete equol in urine (30); 2) *S*-(-) equol is not excreted in the urine or found in the plasma of either newborn infants that lack a developed microflora (53) or in infants up to the age of 4-mo fed exclusively soy infant formula from early life (54,55); 3) incubation of soy, or daidzein with human fecal flora from adults that produce equol, leads to the formation of *S*-(-)equol (36,40); 4) some antibiotics will knock-out the production of equol (18,56,57).

Even though it was known for decades that intestinal bacteria were responsible for the production of *S*-(-)equol, it is only in recent years that specific bacteria capable of converting daidzin/daidzein to *S*-(-)equol have been isolated and identified (Table 1). Interestingly, whereas it was reported that 1.59% of injected [<sup>14</sup>C]genistein was recovered as [<sup>14</sup>C]equol in domestic fowl (77), metabolic and pharmacokinetic studies in humans admin-

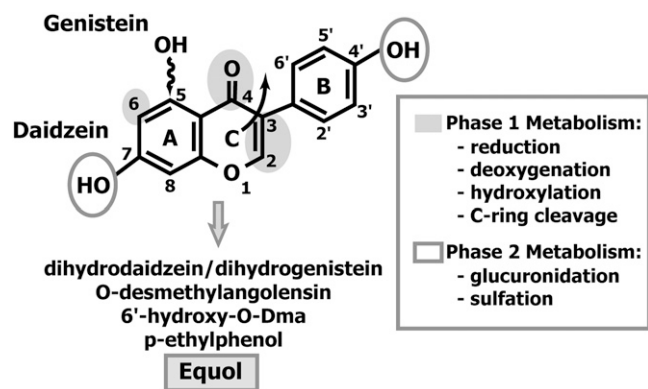
istered [<sup>13</sup>C]daidzein and [<sup>13</sup>C]genistein tracers show conclusively that in humans, *S*-(-)equol is formed from daidzein and not genistein (78). Although the major degradative pathway for genistein leads to *p*-ethylphenol, a phenol first found in goat urine (79) and 4-hydroxyphenyl-2-propionic acid in rats (80), recently it was shown that an anaerobic bacterium from mouse intestine could produce 5-hydroxy-equol from genistein by analogous reactions to those that yield equol from daidzein (66,74). All of these conversions are time dependent and slow, and in humans it takes 12–36 h for the appearance of [<sup>13</sup>C]equol in plasma after oral administration of [<sup>13</sup>C]daidzein (78), which is consistent with a colonic origin for its formation.

Biotransformations that take place after oral administration of soy isoflavones are summarized in Figure 2. The production of *S*-(-)equol from daidzin requires 3 key steps. Daidzin first undergoes hydrolysis to split the glucoside moiety and effect release of the bioavailable aglycon, daidzein. This step is crucial to all soy isoflavones, because the conjugated forms (glucosides) do not cross the enterocyte and are consequently not bioavailable (81). Hydrolysis is very efficient and begins in the proximal intestine by the action of brush border membrane  $\beta$ -glucosidases (82). Bacterial  $\beta$ -glucosidases are also capable of performing this hydrolysis and many of the common bacteria that reside in the intestinal tract do this (62,63,83). The efficiency of this initial hydrolytic step by brush border membrane glucosidases is also exemplified by the very high plasma concentrations of daidzein

**TABLE 1** List of intestinal bacteria that cultured in vitro have been found to biotransform isoflavones to *S*-(-)equol or related intermediates

Reference	Bacterium strain	Source	Reaction <sup>1</sup>
Maruo et al., 2008 (58)	<i>Adlercreutzia equolifaciens</i>	Human	Daidzein → Equol
Minamida et al., 2006 (59)	<i>Asaccharobacter celatus</i> AHU1763	Rat	Daidzein → Equol
Minamida et al., 2008 (60)	<i>Asaccharobacter celatus</i> gen.nov.sp. nov strain do03	Rat	Daidzein → Equol
Ueno and Uchiyama, 2002 (61)	<i>Bacteroides ovatus</i>	Human	Daidzein → Equol
Tsangalis et al., 2002 (62)	<i>Bifidobacterium</i>	Human	Daidzein → Equol
Tsangalis et al., 2002 (62)	<i>Bifidobacterium animalis</i>	Pure culture	Daidzein → Equol
Raimondi et al., 2009 (63)	<i>Bifidobacterium</i> sp (22 strains)	Human	Daidzin → Daidzein
Hur et al., 2000 (64)	<i>Clostridium</i> sp HGH6	Bovine	Daidzin → Dihydrodaidzein
Tamura et al., 2007 (65)	<i>Clostridium</i> -like bacterium	Human	Daidzin → Dihydrodaidzein
Matthies et al., 2008 (66)	<i>Coriobacteriaceae</i> sp MT1B9	Mouse	Daidzein → Equol
Matthies et al., 2008 (66)	<i>Coriobacteriaceae</i> sp MT1B9	Mouse	Genistein → 5-Hydroxy-equol
Wang et al., 2005 (67)	<i>Eggerthella</i> sp Julong 732	Human	Dihydrodaidzein → Equol
Kim et al., 2009 (68)	<i>Eggerthella</i> sp Julong 732	Human	Dihydrodaidzein → Equol
Yokoyama and Suzuki, 2008 (69)	<i>Eggerthella</i> sp YY7918	Human	Daidzein → Equol
Decroos et al., 2005 (70)	<i>Enterococcus faecium</i>	Human	Daidzein → Equol
Tamura et al., 2007 (65)	<i>Escherichia coli</i> (HGH21 and HGH6)	Human	Daidzin → Daidzein
Yu et al., 2008 (71)	<i>Eubacterium</i> sp D1 and D2	Pig	Daidzein → Equol
Decroos et al., 2005 (70)	<i>Finigoldia magna</i>	Human	Daidzein → Equol
Decroos et al., 2005 (70)	<i>Lactobacillus mucosae</i>	Human	Daidzein → Equol
Wang et al., 2006 (72)	<i>Lactobacillus</i> sp Niu-O16	Human	Daidzein → Equol
Ishimi et al., 2008 (73)	<i>Lactococcus garvieae</i> (Lc 20-92)	Human	Daidzein → Equol
Ueno and Uchiyama, 2002 (61)	<i>Ruminococcus productus</i>	Human	Daidzein → Equol
Matthies et al., 2009 (74)	<i>Slackia</i> sp HE8	Human	Daidzein → Equol
Matthies et al., 2009 (74)	<i>Slackia</i> sp HE9	Human	Genistein → 5-Hydroxy-equol
Jin et al., 2009 (75)	<i>Slackia equolifaciens</i> (Strain DZE)	Human	Daidzein → Equol
Jin et al., 2008 (76)	Strain PUE	Human	Purarin → Daidzein
Ueno and Uchiyama, 2002 (61)	<i>Streptococcus intermedius</i>	Human	Daidzein → Equol
Decroos et al., 2005 (70)	<i>Veillonella</i> sp	Human	Daidzein → Equol
Decroos et al., 2005 (70)	Mixture of <i>Lactobacillus mucosae</i> EP12, <i>Enterococcus faecium</i> EP11, <i>Finigoldia magna</i> EP13, and <i>Veillonella</i> sp	Human	Daidzein → Equol

<sup>1</sup> In most cases the definitive structure of the equol product was not determined, but it can be assumed that in all cases the product is the *S*-(-)equol enantiomer.



**FIGURE 2** Principal metabolic biotransformations of the soy isoflavones daidzein and genistein. The structure for daidzein is shown and genistein has the identical structure but with an additional hydroxyl group at position C-5 of the A-ring, highlighted by the wavy arrow.

and genistein in infants fed soy infant formula (54,55) that have immature and undeveloped gut microflora (53). Daidzein is reduced to *S*-(-)equol through the intermediate dihydrodaidzein then converted by deoxygenation to yield *S*-(-)equol. Daidzein is also metabolized to O-desmethylangolensin as a result of cleavage across the heterocyclic ring (84), but this metabolite appears to be of little interest in that it has no known biological activity.

Bacteria are enantioselective in metabolizing daidzein to exclusively *S*-(-)equol and not *R*-(+)-equol (40,67,72). It is not completely clear whether the conversion of daidzein to *S*-(-)equol is performed by a single bacterium or whether there are distinctly different bacteria that execute these reactions, or both. The large variability in the levels of dihydrodaidzein and *S*-(-)equol in human urine (85) would indicate that there is more than a single bacterium responsible for producing *S*-(-)equol. Furthermore, the finding that certain antibiotics selectively inhibit the formation of equol but not dihydrodaidzein when human feces from equol-producers are incubated with daidzein also supports this contention (56). The list of intestinal bacteria that can produce equol in culture is ever increasing (Table 1); a number of strains have been isolated that perform only conversion of daidzin/daidzein to dihydrodaidzein whereas others appear to be able to completely convert daidzein to equol (Table 1). One in particular, *Lactococcus garvieae*, that is found in some Italian cheeses (51) and has been isolated from human feces (61,73) was shown to efficiently convert daidzin to *S*-(-)equol and has been used to produce the first natural *S*-(-)equol-containing nutraceutical (52,86). Whether demonstrating formation of *S*-(-)equol in culture can be considered representative of intraluminal colonic formation of *S*-(-)equol remains uncertain. It may, e.g. under specific conditions be possible to demonstrate conversion of daidzein to equol in vitro, yet such conditions may not necessarily be reflective of the intraluminal milieu in the human intestinal tract. For example, culturing human fecal flora from equol-producers under conditions that enhance fermentation, such as a high nonstarch polysaccharide medium, enhances conversion of daidzein to equol (87), as do hydrogen gas, butyrate, and propionate (59,70). Despite the ability to manipulate equol production in vitro, human dietary intervention studies using prebiotics or probiotics have had little impact on its formation (88–92). Therefore, some caution is required in relying on in vitro fecal cultures from adults as a means of confirming equol-producer status (93). Such approaches should always be coupled with specific measurement

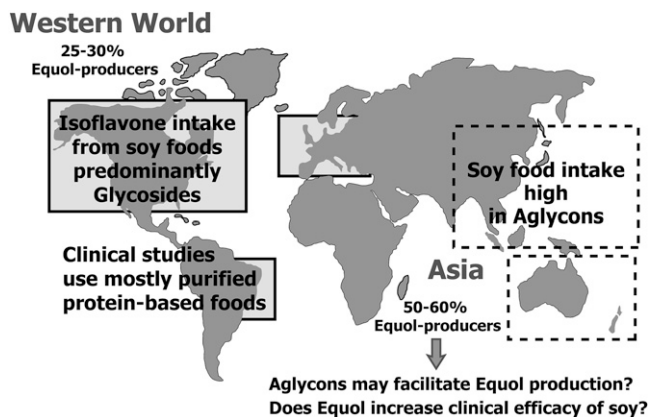
of equol in urine (94,95). In the future, it is likely that molecular techniques in bacteriology may help to more accurately define equol-producer status.

### Frequency of equol-producers

All animal species tested produce equol in response to consumption of isoflavones whether from soy protein or clover (16,23,24). Rodents in particular have very high plasma *S*-(-)equol concentrations, in part because most commercial rodent diets are formulated with soy protein (20,96). For this reason, careful consideration is needed when using rodents, particularly if the primary endpoints being examined are in any way influenced by estrogens, either directly or indirectly, through upstream signaling pathways with estrogen response elements (20,97–99). Frequently, there is a lack of awareness on the part of investigators of the type and composition of the diets being used by their animal facilities or by the vendors providing rodents for research studies. A high batch-to-batch variability in the isoflavone content of commercial rodent unpurified diet makes it impossible to control for the background level of these phytoestrogens (97).

When the association between soy isoflavones and equol was first made, it was noted that not all healthy adults produced equol when challenged with soy protein (36). The first reported dietary intervention study of the 6 healthy adults fed 40 g of textured vegetable protein for 7 consecutive days found only 4/6 excreted equol in urine; the term equol-producers was thus coined. Many other studies of larger sample sizes have provided a consensus that only 25–30% of the adult population of Western countries produce *S*-(-)equol when fed soy foods containing isoflavones (94,100,101). This is significantly lower than the reported 50–60% frequency of equol-producers in adults from Japan, Korea, or China (102–105) or in Western adult vegetarians (95). The reasons for these differences are unclear but are important to understand if the hypothesis that the ability to produce equol when consuming soy foods (equol hypothesis) is advantageous in terms of enhancing health benefits can be clearly demonstrated (24,106). Thus far, the data are inconclusive, but this is partly because, to our knowledge, none of the clinical studies have preselected participants on the basis of equol-producer status but rather have retrospectively subanalyzed data from equol-producers, and most are underpowered. Significant differences in gene expression between equol-producers and equol nonproducers have been demonstrated in postmenopausal women exposed to an isoflavone supplement, with the most notably significant alterations in expression of a number of estrogen-responsive genes (107).

It would appear that equol-producer status is a relatively stable phenomenon, as evidenced from repeat testing over prolonged periods of time in adults (24,89,95,108). There have been many studies looking at associations between equol-producer status and dietary components, including fat and carbohydrate composition (93,101), PUFA (109), dairy intakes (110), lactose (111), green tea consumption (112), seaweed (113), and soy food intake (103,105,110), but no clear conclusions can be made. Prolonged soy food consumption appears not to be a factor driving equol formation (114). One possible explanation for the differences in the frequency of equol-producers among populations, we speculate, may be related to the type of soy foods consumed (115). There are marked differences in the isoflavone composition of Western and Asian soy foods (Fig. 3) (41). Asians consume a high proportion of isoflavone aglycons, because fermented soy foods account for



**FIGURE 3** Differences in the type of soy foods consumed by Western and Asian populations may account for differences in the frequency of equol-producers.

about one-third of the total intake of soy foods (116,117). Based upon the typical 20–50 mg/d intake of total isoflavones by Asians, we estimate that 10–30 mg are ingested in the form of aglycons, which in most adults are absorbed faster than glycosides (43,102,118–121) and may be more easily converted to equol than glycosides. In a recent cholesterol-lowering dietary intervention study of a soy germ-enriched pasta containing predominantly isoflavones in the aglycon form, it was found that 69% of the patients were equol-producers (115), which is considerably higher than the 20–30% expected frequency for Westerners (95). Most dietary intervention studies of soy have used foods or products made from isolated soy proteins as the source of isoflavones (122) or soy isoflavone supplements that contain almost exclusively isoflavone glucosides (119). Isolated soy proteins are not commonly consumed in the Asian diet and this may explain the lower frequency of equol-producers in Western adults. Furthermore, if equol production does enhance the efficacy of soy foods (24,106), then this could explain the variable results obtained from recent studies of soy foods. Future studies of foods or supplements containing predominantly isoflavone aglycons may be more meaningful and should be considered as more closely mimicking the isoflavone composition of a typical Asian diet (115).

Although the type of soy foods consumed may influence equol production, the overriding factors governing equol production remain: 1) the requirement of specific equol-producing bacteria; 2) the presence of a source of substrate(s), which for soy is daidzin or daidzein; and 3) having optimum intraluminal conditions in terms of redox potential for the reactions to take place. Clearly, if the bacteria are lacking, then it is not possible to produce equol, but the equol-producing bacteria may also be present but inactive. It is only possible to be an equol-producer when consuming soy foods or products containing isoflavones. The very low urinary or plasma equol concentrations reported in many large population-based studies of Westerners (123–127) are merely an indication of the fact that soy foods are not consumed on a regular basis, rather than a reflection of the true frequency of equol-producers in these populations. Therefore, to tease out an equol-producer, it is crucial to challenge with sufficient amounts of substrate for conversion to equol and it is necessary to allow sufficient transit time for the substrate to reach the distal gastrointestinal tract and colon where the bacterial reactions take place. These practical considerations have been discussed in detail previously (94,95). The standard

approach is to feed a soy isoflavone-containing food, such as soymilk, or an isoflavone supplement, such as soy germ, that is preferentially high in daidzein (128) for 3 consecutive days to attain a steady state (94,95,108,129). Urine is then collected and daidzein and S-(–)equol measured. We recently opted to define an equol-producer based on the ratio of equol:daidzein (95), because this reflects the product/precursor relationship and overcomes the drawbacks of using absolute urinary (or plasma) equol concentrations (36,94,101,130), which are subject to considerable variation due to inter-individual differences in isoflavone pharmacokinetics (78,102,119,120,131–133). Defining an equol-producer by the equol:daidzein ratio also circumvents the need for accurately timed 24-h urine collections.

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