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Microbial Host Interactions

Microbial equol production attenuates colonic methanogenesis and sulphidogenesis *in vitro*

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ABSTRACT

Hydrogen gas produced during colonic fermentation is excreted in breath and flatus, or removed by hydrogen-consuming bacteria such as methanogens and sulphate-reducing bacteria. However, recent research has shown that H_2 is also consumed by equol-producing bacteria during the reduction of daidzein into equol. In this study, the interactions between methanogens, sulphate-reducing, and equol-producing bacteria were investigated under *in vitro* simulated intestinal conditions. In the presence of daidzein, the equol-producing bacterial consortium EPC4 gave rise to equol production in cultures of *Methanobrevibacter smithii* or *Desulfovibrio* sp. as well as in faecal samples with methanogenic or sulphate-reducing abilities. Moreover, this supplementation significantly ($P < 0.001$) decreased the methanogenesis and sulphidogenesis. The attenuation did not occur in the absence of a daidzein source. Additionally, there was no influence of soy germ powder, daidzein or equol as such, excluding a possible inhibition by these compounds. Finally, a stronger decrease was observed with increasing amounts of EPC4 and a constant equol production, suggesting that the observed effect was only partly caused by the action of daidzein as a hydrogen sink. These findings are of relevance since abdominal discomfort such as bloating and flatulence, are related to colonic gas production, whereas equol has potential health benefits.

1. Introduction

Phyto-oestrogens are polyphenolic non-steroidal secondary plant metabolites with pro- and/or anti-oestrogenic properties and potential health effects. The major dietary phyto-oestrogens are genistein and daidzein which are isoflavones present in soy (*Glycine max* (L.) Merr.). Although soy consumption results in increased levels of the parent isoflavones in plasma and urine, these compounds can also be metabolised before, during or after initial intestinal absorption and both human and microbial transformation reactions can occur [1]. Genistein is converted to 6'-hydroxy-*O*-desmethylangolensin, 2,4,6-trihydroxybenzoic acid and *p*-ethyl phenol [2–4], whereas the major microbial metabolites of daidzein are equol and *O*-desmethylangolensin [2,5].

Equol has gained a lot of attention since the postulation of the equol hypothesis, contending a greater efficacy of soy food diets in so-called equol producers [6]. This metabolite is *in vitro* more bio-active than its precursor daidzein: it has a higher oestrogenicity [7–11], is a more potent anti-oxidant [12–15], and possesses anti-androgenic properties [16]. Additionally, equol has a higher effective free fraction circulating in human serum [17] and a slower plasma clearance [6] compared to daidzein. However, equol is not of plant origin and is exclusively formed by the intestinal microbiota [18–21]. Approximately one third of the Caucasians harbour an intestinal microbial ecosystem supporting the conversion of daidzein into equol [22] and 75 years after the first description of this molecule by Marrian and Haslewood [23], the factors triggering this process remain largely unknown [24]. Although Atkinson et al. [22] summarised that there is conflicting evidence available regarding the potential health benefits associated with the ability to produce equol, there is a growing interest in dietary applications enhancing equol production in humans.

Decroos et al. [25] isolated and characterised a microbial equol-producing consortium (EPC4), originating from a human faecal sample. This co-culture consists of four dominant bacterial strains, identified as *Enterococcus faecium* EPI1, *Lactobacillus mucosae* EPI2, *Finegoldia magna* EPI3, and a *Veillonella* sp. related strain EP. Administration of EPC4 to the simulator of the human intestinal

Abbreviations: EPC4, equol-producing consortium; SHIME, simulator of the human intestinal microbial ecosystem.

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microbial ecosystem (SHIME), a dynamic *in vitro* model of the gastro-intestinal tract [26], inoculated with a non-equol-producing faecal sample, resulted in the production of equol in the distal colon parts without adverse effects on the general composition and activity of the microbial community [27]. This indicates that EPC4 can survive in a complex microbial ecosystem and maintains its metabolic activity. It was found that equol production was stimulated to a large extent by hydrogen gas, probably acting as electron donor in this biotransformation [25]. Hydrogen gas is formed in the colon by a variety of hydrolytic and saccharolytic bacteria to dispose reducing equivalents during fermentation [28]. In humans, part of the H_2 is excreted by breath and flatus, but the most important way of hydrogen disposal is interspecies H_2 transfer [29]. Fermentative H_2 is consumed *in situ* by hydrogenotrophic microorganisms, mainly methanogenic archaea, homo-acetogenic and, sulphate-reducing bacteria. There is typically a competition between sulphate-reducing bacteria and methanogens for their common substrate (H_2) [30], but the relationship between these microorganisms seems to be more complex than previously suggested [31]. On the other hand, the equol-producing activity of EPC4 may also interact with the H_2 consumption of these hydrogenotrophic microorganisms. Therefore, the aim of this study was to investigate the interplay between methanogens, sulphate-reducing, and equol-producing bacteria under conditions simulating the human colon.

2. Materials and methods

2.1. Microbial cultures and growth conditions

Methanobrevibacter smithii DSM 2375 and *Desulfovibrio* sp. DSM 7057 were obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) and grown at 37 °C for 48 h in a modified Balch I medium [32], under an atmosphere of $N_2/H_2/CO_2$ (65/20/15). Two g l^{-1} of Na_2SO_4 were added to the medium of *Desulfovibrio* sp.. EPC4 was grown at 37 °C for 48 h in brain heart infusion (37 g l^{-1}; Oxoid, Drongen, Belgium) supplemented with 0.5 g l^{-1} cysteine-HCl.H_2O under an atmosphere of $N_2/H_2/CO_2$ (65/20/15) [25].

Faecal cultures, derived from samples of healthy post-menopausal Caucasian women (age 57 ± 6 years; BMI 23.8 ± 3.7 kg m^{-2}), were prepared and phenotyped as poor, moderate, or strong equol producers following the protocol of Bolca et al. [24]. The donors had no history of gastro-intestinal disease, had not taken antibiotics recently (3 months) nor consumed soy or soy-based products at least 5 days prior to sample delivery. Ethical approval was granted from the Ethics Committee of the Ghent University Hospital (EC UZG 2004/044).

Culture conditions supporting the growth of EPC4 and methanogens or sulphate-reducing bacteria simultaneously and mimicking the environment of the distal colon, were obtained by supplementing autoclaved fluid from the colon descendens compartment of a dynamic *in vitro* model of the human gastro-intestinal tract (SHIME [26]) with 0.1 g l^{-1} Na_2SO_4 under an atmosphere of $N_2/H_2/CO_2$ (65/20/15). Three different test media were prepared by adding (i) 0.2 g l^{-1} soy germ powder (SoyLife™micro25, Frutarom Netherlands BV, Veenendaal, The Netherlands), (ii) 2 mg l^{-1} daidzein (Sigma-Aldrich, Bornem, Belgium), or (iii) 2 mg l^{-1} equol (Extrasynthèse, Genay Cedex, France) to this control medium. The first test medium with soy germ powder contained 7.59 ± 0.12 μM daidzein aglycon equivalents, the second 7.65 ± 0.08 μM daidzein aglycon equivalents as daidzein, and the third 7.75 ± 0.12 μM daidzein aglycon equivalents as equol.

2.2. Experiment 1: interactions between EPC4 and Methanobrevibacter smithii or Desulfovibrio sp.

In these experiments, actively growing bacterial suspensions containing 1.2×10^6 cells ml^{-1}, were used. The inoculum was a combination of EPC4 with either *Methanobrevibacter smithii* or *Desulfovibrio* sp. in different proportions, yielding a mixture containing 0, 10, 50, 90, and 100% (v/v) EPC4. The control and test medium with soy germ powder were inoculated with 5% (v/v) inoculum. After 4 days of incubation at 37 °C, samples were taken for headspace analyses and quantification of the equol production.

2.3. Experiment 2: interactions between EPC4 and non-equol-producing faecal cultures with methanogenic or sulphate-reducing abilities

Six faecal cultures unable to convert daidzein into equol, were selected for these experiments. Three of them (M1, M2, M3) were from methane producers, whereas S1, S2, S3 originated from participants without CH_4 in their breath samples. The faecal inocula were used as such or first enriched with 1% (v/v) EPC4. The control and the three different test media were inoculated with 5% (v/v) inoculum. Samples for headspace analyses, quantification of the equol production, and determination of the cell density and short-chain fatty acid profile, were taken after 4 days of incubation at 37 °C.

2.4. Analytical methods

2.4.1. Enumeration of bacterial cells

The bacterial cell density was determined with a 50 mW sapphire solid-state diode laser (488 nm) as excitation light source, after staining with 0.5 μM Syto16 (Invitrogen, Merelbeke, Belgium) and addition of a known amount of polystyrene microspheres (CytoCount beads, DakoCytomation, Heverlee, Belgium), by flow cytometry (CyAn™, DakoCytomation, Heverlee, Belgium) as described by Verthé and Verstraete [33].

2.4.2. Extraction and quantification of isoflavones

The isoflavones were extracted by liquid/liquid extraction with diethyl ether as solvent [24]. Quantitative analyses of daidzein and the metabolites dihydrodaidzein, *O*-desmethylangolensin and equol were performed by HPLC-UV using a Waters 2695 Alliance separations module and 996 PDA detector (Waters, Milford, Massachusetts, USA) [34]. The reversed-phase column was an XTerra™ MS C_{18} (5 μm, 4.6 mm × 250 mm; Waters, Milford, Massachusetts, USA). UV detection was performed at the wavelength corresponding to the absorption maximum of each analyte: 248 nm for daidzein, 277 nm for dihydrodaidzein and *O*-desmethylangolensin, and 230 nm for equol. Calibration curves were constructed using analytical standards (daidzein was purchased from Sigma-Aldrich (Bornem, Belgium), equol from Extrasynthèse (Genay Cedex, France) and dihydrodaidzein and *O*-desmethylangolensin from Plantech UK (Reading, UK)).

2.4.3. Short-chain fatty acid determination

The short-chain fatty acids (acetic, propionic, butyric, isobutyric, isovaleric, valeric, capronic, and isocapronic acids) were extracted with diethyl ether and determined with a Di200 GC-FID (Shimadzu, 's Hertogenbosch, The Netherlands) with a free fatty acid packed EC-1000 Econo-Cap column (1.2 μm, 0.53 mm × 25 m; Alltech, Laarne, Belgium) [35].

2.4.4. Gas measurements

End-expiratory breath samples were collected using the Quin-Tron GaSampler system (Ecce Medical, Schoten, Belgium) and