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ORIGINAL PAPER

Karel Decroos · Steffi Vanhemmens · Sofie Cattoir
Nico Boon · Willy Verstraete

Isolation and characterisation of an equol-producing mixed microbial culture from a human faecal sample and its activity under gastrointestinal conditions

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Abstract Only about one third of humans possess a microbiota capable of transforming the dietary isoflavone daidzein into equol. Little is known about the dietary and physiological factors determining this ecological feature. In this study, the in vitro metabolism of daidzein by faecal samples from four human individuals was investigated. One culture produced the metabolites dihydrodaidzein and *O*-desmethylangolensin, another produced dihydrodaidzein and equol. From the latter, a stable and transferable mixed culture transforming daidzein into equol was obtained. Molecular fingerprinting analysis (denaturing gradient gel electrophoresis) showed the presence of four bacterial species of which only the first three strains could be brought into pure culture. These strains were identified as *Lactobacillus mucosae* EPI2, *Enterococcus faecium* EPI1 and *Finegoldia magna* EPI3, and did not produce equol in pure culture. The fourth species was tentatively identified as *Veillonella* sp strain EP. It was found that hydrogen gas in particular, but also butyrate and propionate, which are all colonic fermentation products from poorly digestible carbohydrates, stimulated equol production by the mixed culture. However, when fructo-oligosaccharides were added, equol production was inhibited. Furthermore, the equol-producing capacity of the isolated culture was maintained upon its addition to a faecal culture originating from a non-equol-producing individual.

Keywords Isoflavone · Equol · Hydrogen gas · Bacteria · FOS

K. Decroos · S. Vanhemmens · S. Cattoir · N. Boon
W. Verstraete (✉)
Laboratory of Microbial Ecology and Technology (LabMET),
Ghent University, Coupure Links 653,
9000 Ghent, Belgium
E-mail: willy.verstraete@UGent.be
Tel.: +32-9-2645976
Fax: +32-9-2646248

Introduction

Over the last two decades there has been much interest in the isoflavonoids, diphenolic compounds abundant in soy (Axelson et al. 1984; Peters et al. 1986; Phillips 1992), because of their beneficial effects on human health. They have structural similarities to the human female hormone, 17β-estradiol, and other steroid hormones, and can bind to estrogen receptors in many tissues, acting as a weak agonist or antagonist (Kuiper et al. 1997). Several authors report their potential role in the chemoprevention and therapy of hormone-related diseases such as breast cancer and osteoporosis (Coward et al. 1993; Messina et al. 1994; Adlercreutz 1995). An important issue with respect to the bioavailability and bioactivity of the isoflavonoids is their metabolic fate after ingestion, in particular their microbial transformation in the colon (Kurzer and Xu 1997). Equol, an intestinal metabolite of daidzein, in particular has gained much attention, as its appearance is considered as being beneficial for health (Setchell et al. 2002).

Daidzein (4′,7-dihydroxyisoflavone) is one of the principal isoflavonoids (up to 1% of dry weight in soy germs) (Murphy et al. 2002). In soybeans and non-fermented soy products it occurs only in small amounts as the aglycone (less than 5% of total daidzein), but mainly as a non-absorbable, biologically inactive glycoside, acetylglycoside or malonylglycoside (Wang and Murphy 1994). Upon ingestion, the latter are partially hydrolysed to the aglycone in the small intestine, followed by absorption through the gut epithelium (Day et al. 2000). A considerable fraction is neither hydrolysed nor absorbed in the small intestine and reaches the colon, together with an amount that is excreted at the end of the small intestine through enterohepatic circulation. In the colon, the glycosylated, sulfated and glucuronidated forms of daidzein are deconjugated by bacterial enzymes (Day et al. 2000; Richelle et al. 2002; Chen et al. 2003; Rowland et al. 2003; Setchell et al. 2003). The aglycones

are then absorbed or subjected to further metabolism by the intestinal bacteria (Wiseman 1999). Under the reducing conditions of the colon it has been proposed that daidzein is first transformed (Fig. 1) into dihydrodaidzein (DHD) and from there further into *O*-desmethylangolensin (*O*-DMA) or equol (Chang and Nair 1995; Heinonen et al. 1999). Equol is exclusively produced by the intestinal bacteria (Bowey et al. 2003). The composition of the microbiota is a determining factor for the nature of the metabolites formed, and is variable among individuals (Xu et al. 1995; Rafii et al. 2003; Atkinson et al. 2004). Only approximately one third of humans harbour an intestinal microbiota capable of transforming daidzein into equol (equol producers); *O*-DMA production is spread more widely and is not directly related to equol (Kelly et al. 1995; Rowland et al. 2000; Lampe et al. 2001; Setchell et al. 2002).

Equol has a higher binding affinity to human estrogen receptors α and β than daidzein (10–80 times) and induces transcription more strongly than any other isoflavone (Sathyamoorthy and Wang 1997; Kuiper et al. 1998; Morito et al. 2001; Kostelac et al. 2003; Muthyala et al. 2004). It is also reported to be a stronger antioxidant than the latter (Mitchell et al. 1998) and recently, equol has been found to exhibit anti-androgenic activities, inhibiting prostate cancer development (Lund et al. 2004). Equol is absorbed more efficiently through the colon wall than daidzein, has a slower plasma clearance than its precursor, and is metabolically inert (Setchell et al. 2002).

The biological properties of equol suggest that the efficacy of a diet containing soy might be dependent on the equol-producing status of the individual. Recent reports seem to support this (Akaza et al. 2004; Meyer et al. 2004). There is a growing interest in dietary applications which can modulate the equol production in the colon. However, the specific bacterial species and environmental conditions in the colon involved in the production of this important metabolite are yet to be discovered. Although equol production has been established in vitro from human faecal samples (Rafii et al. 2003; Atkinson et al. 2004), efforts to isolate bacteria that produce equol have not been successful so far. They have resulted in the isolation of strains that were able to transform daidzein into DHD and *O*-DMA (Hur et al. 2000; Hur et al. 2002; Schoefer et al. 2002).

This study presents the isolation of a stable mixed microbial culture from a human faecal sample that is capable of transforming daidzein into equol. Furthermore, the influence of certain environmental conditions that occur in the colon, such as the presence of colonic end-products of carbohydrate fermentation [hydrogen gas and short chain fatty acids (SCFAs)] and fructo-oligosaccharides (FOS), on daidzein metabolism by this culture were investigated. Finally, we assessed equol production after supplementation of the isolated culture with a faecal culture from a non-equol-producing individual.

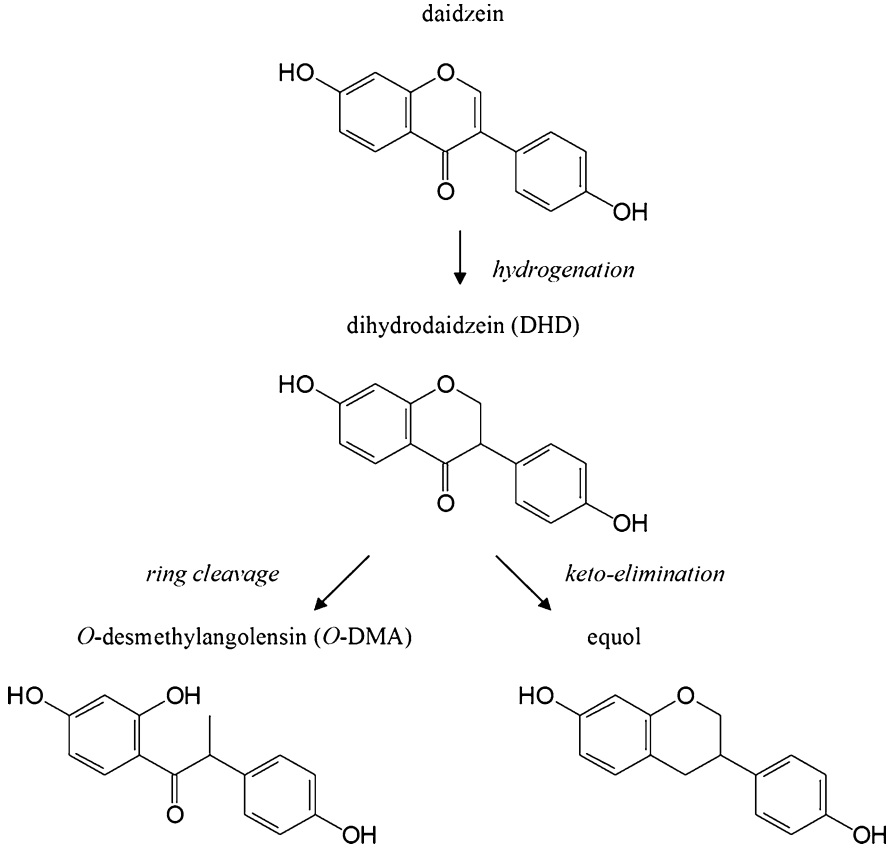

Fig. 1 Proposed pathway for the reductive metabolism of daidzein by intestinal bacteria

Materials and methods

Faecal samples

Faecal samples from four human volunteers were very kindly supplied by Dr. Barbara Meyer (University of Wollongong, Australia) and randomly chosen from participants from a previous study (Meyer et al. 2004). Five grams of freshly voided faecal sample was homogenised and suspended in 50 ml of an autoclaved 50 mM phosphate buffer at pH 7 supplemented with 1 g sodium thioglycolate/l (filter-sterilised). Particulate material was removed by centrifugation at 500 *g* for 1 min and after addition of 20%(v/v) glycerol, samples were stored at −80°C.

Equol production by the faecal cultures

Bacterial cells were retrieved from 10 ml frozen cultures by centrifugation at 3,000 *g* for 3 min. After washing three times with sterile saline (8.5 g sodium chloride/l) the cells were transferred to penicillin flasks containing 50 ml autoclaved brain heart infusion (Oxoid, Drongen, Belgium) supplemented with 0.5 g L-cysteine/l (BHI_a) and 200 μmol daidzein/l. The flasks were made anaerobic by flushing the headspace with nitrogen gas for 20 min. The samples were incubated at 37°C for 72 h and daily samples were taken for HPLC analysis.

Isolation of bacterial strains and equol production assay

An amount of 100 μl of a frozen culture was inoculated into penicillin flasks containing 10 ml BHI_a. After flushing the headspace with nitrogen gas for 20 min, the flasks were incubated at 37°C for 48 h. Decimal serial dilutions in sterile saline were plated on BHI_a agar and incubated in an anaerobic jar under an atmosphere of nitrogen/hydrogen/carbon dioxide (84/8/8) at 37°C for 72 h. Single colonies were transferred to penicillin flasks containing 10 ml growth medium [BHI_a or De Man Rogosa Sharpe (MRS) broth (Oxoid, Drongen, Belgium)] supplemented with 100 μmol daidzein/l. The flasks were made anaerobic and after 72 h of incubation at 37°C samples were taken for HPLC analysis.

Bacterial strains

The following strains were obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany), *Veillonella parvula* DSM 2008, *V. parvula* ssp *rodentium* DMS 20373 and *V. alcalescens* ssp *ratti* DSM 20376. They were grown at 37°C in a medium containing 5 g trypticase, 3 g yeast extract, 7.5 g sodium (DL) lactate, 0.75 g Tween 80, 1 g glucose, 0.5 g sodium thioglycolate and 3 mg putrescine per litre distilled water, under an atmosphere of N_2 gas.

Enumeration of the total number of anaerobic bacteria

One ml of each decimal dilution series of the culture, prepared in physiological solution, was transferred to pouring plates containing BHI_a medium to which 15 g agar/l was added. The plates were incubated in an anaerobic jar under a atmosphere of nitrogen/hydrogen/carbondioxide (84/8/8) at 37°C and after 72 h colonies were enumerated.

Influence of H_2 and SCFAs on daidzein metabolism

An amount of 100 μl of thawed EPC4 (see Results section) transferred in 10 ml BHI_a and incubated under a nitrogen atmosphere at 37°C for 48 h served as the inocolum. The test suspension consisted of 2%(v/v) inoculum in 50 ml BHI_a with 100 μmol daidzein/l to which 15 g sodium acetate, propionate or butyrate/l (filter-sterilised) was added. No organic salt was added to the control. The suspensions were prepared in 100 ml penicillin flasks and the headspace was flushed with nitrogen gas for 20 min; subsequently the headspace was filled with nitrogen or hydrogen gas and finally incubated at 37°C for 6 days. The different treatments are referred to as control, AC, PR, BT and control-H_2, AC-H_2, PR-H_2 and BT-H_2 respectively, being the test suspensions to which either nothing, sodium acetate, propionate or butyrate was added, with the headspace filled with nitrogen or hydrogen gas respectively. Daily samples were taken for HPLC analysis and the enumeration of the total anaerobic bacteria counts. The different treatments were executed in triplicate, and data were compared using Student's *t*-test.

Equol production in the presence of Pd^0

One ml of a 48 h-old culture of EPC4 was transferred into a penicillin flask containing 50 ml BHI_a supplemented with 100 μmol daidzein/l to which 10 g pulverised palladium (Pd^0)/l was added. The head-space was flushed with N_2 and the culture was incubated at 37°C for 2 weeks. Every 48 h samples were taken for HPLC analysis and bacterial enumeration. No Pd^0 was added to the control. The experiment was conducted in triplicate.

Influence of fructo-oligosaccharides on equol production

A 48 h old culture of EPC4 was transferred to a penicillin flask containing 50 ml BHI_a supplemented with 100 μmol daidzein/l and 10 g/l of a FOS mixture with an

average polymerisation degree of 10 (Cosucra NV, Warcoing, Belgium). The headspace was flushed with N_2 and the mixture was incubated at 37°C for 2 weeks. Every 48 h samples were taken for HPLC analysis. No FOS was added to the control. The experiment was conducted in triplicate.

Supplementation of EPC4 with a non-equol-producing faecal culture

The faecal cultures of samples 1 and 2 (Table 1) and EPC4 were inoculated in BHI_a at 37°C under anaerobic conditions. After 48 h EPC4 was combined with either sample 1 or 2 in different proportions to a total volume of 50 ml, so that the total mixture contained 0, 1, 5, 25, 50, 75 and 100%(v/v) EPC4. The mixtures were washed with sterile saline, resuspended in 50 ml BHI_a supplemented with 100 µl daidzein, and incubated under N_2 at 37°C for 2 weeks. Every 48 h, samples were taken for HPLC analysis. This experimental set-up was repeated with addition of 10 g FOS/l (see above) to the final medium. The experiment was conducted in triplicate.

HPLC analysis

One ml of each sample was extracted three times with 1 ml diethylether, the ether fractions were combined, evaporated to dryness under a stream of nitrogen gas at 37°C and redissolved in 500 µl of 50%(v/v) ethanol:dimethylsulfoxide and stored at 4°C until analysis.

HPLC analysis was performed with a Dionex HPLC (Sunnyvale, Calif., USA) with an autosampler ASI-100, pump series P580, STH585 column oven and UV-VS detector UVD340S. A 20 µl volume of the sample was injected and separated over a Genesis C18 column (150×4.6 mm, 4 µm) (Jones Chromatography, UK). The temperature was set at 25°C and the flow rate was maintained at 1 ml/min. Elution was isocratic with a mobile phase consisting of methanol:acetonitrile:water:acetic acid (33.3:16.7:50:0.1). Equol was detected at 205 nm; daidzein, DHD and *O*-DMA at 260 nm. UV-absorption spectra of the peaks, which allowed identification of the peaks after comparison with pure standards, were recorded by performing a wavelength scan between 200 and 600 nm. Recording and processing of the chromatograms was performed using the Chromeleon software package (Dionex, Sunnyvale, Calif., USA). Calibration curves for the quantification of daidzein and its metabolites were constructed using pure standards obtained from Plantech UK (Reading, UK). The extraction efficiency was defined as the percentage daidzein or equol that could be recovered after addition of a known amount of these compounds to BHI_a.

Table 1 Concentrations of daidzein (*Da*), *O*-DMA and equol (*Eq*) (µmol/l) during fermentation of BHI_a, supplemented with 200 µmol daidzein/l, with faecal cultures of four volunteers. *ND* not detected, – not determined; DHD was detected in only samples 2 and 4; O-DMA and equol in samples 2 and 4, respectively

Fermentation time (h)	Sample 1	Sample 2			Sample 3	Sample 4		
	Da	Da	DHD	*O*-DMA	Da	Da	DHD	Eq
0	197	194	0	0	192	199	0	ND
24	–	–	–	–	–	98	15	27
48	136	55	17	50	160	43	18	64
72	127	35	8	60	154	2	9	97

DNA extraction, PCR and denaturing gel electrophoresis

DNA of the mixed and pure cultures was extracted according to the method of Boon et al. (2000) and denaturing gel elecrophoresis (DGGE) analysis, using universal bacterial primers, was performed according to Possemiers et al. (2004).

Cloning and identification of bacterial species

The 16S rRNA genes from EPC4 and the pure isolated strains were cloned using a TOPO-TA cloning kit (Invitrogen, Carlsbad, Calif., USA) according to the manufacturer's instructions. Sequencing of the partial 16S rDNA fragments was performed by IIT Biotech (Bielefeld, Germany).

Analysis of DNA sequences and sequence identity searches were completed with standard DNA sequencing programs and the BLAST server of the National Center for Biotechnology Information using the BLAST algorithm (Altschul et al. 1997) and BLASTN program for the comparison of a nucleotide query sequence against a nucleotide sequence database.

Results

Equol production by the faecal cultures

The quantitative data on the daidzein metabolism by the four faecal cultures are summarised in Table 1. Microbial metabolites of daidzein were found in samples 2 and 4. In sample 4 practically all of the daidzein was transformed into equol and DHD and there were no traces of *O*-DMA. In sample 2 the metabolites DHD and *O*-DMA were formed during incubation and no equol was found. The daidzein concentration decreased in samples 1 and 3 by about 30% during the 72 h of incubation. However, in these samples, no DHD, equol or *O*-DMA, microbial metabolites of daidzein, could be detected.

The HPLC elution profiles (Fig. 2a) gave a qualitative and quantitative image of the daidzein metabolism by the bacteria in these samples. The identity of DHD, equol and *O*-DMA was confirmed after comparing the

retention times and UV-absorption spectra of the analysed compounds and commercially purchased DHD, equol and *O*-DMA respectively (Fig. 2b). The extraction efficiency of daidzein and equol was 97.7 ± 1.3%.

Isolation and characterisation of an equol-producing mixed culture

By picking colonies from plates on which serial dilutions from the equol-producing faecal sample had grown, one colony was retrieved that produced equol upon subculturing. Light microscopy (Polyvar, Basel, Switzerland) revealed several cell morphologies within this colony. The mixed nature of the culture was confirmed by DGGE analysis (Fig. 3). After 1 day of incubation in BHI_a two bands were dominant; after 5 days in the same medium five dominant bands were found. When grown for 5 days in MRS four bands appeared. Equol production began only after 4 days of incubation in BHI_a medium. No daidzein metabolism was observed during the whole incubation period when the culture was grown in MRS. A stock of this culture was made in BHI_a by adding 20%(v/v) glycerol, and was stored at −80°C.

By further diluting and replating this mixed culture, three colonies with different morphologies were harvested. These subcultures contained a single species, as determined by light microscopy. The DGGE analysis confirmed this finding and demonstrated the presence of the three isolates as dominant bands in the mixed culture (Fig. 3). The identity of the isolated strains was determined by comparing the sequence of the 16S rRNA gene with a database. The first isolate had 99% sequence similarity with *Enterococcus faecium* and was named *E. faecium* strain EPI1. A second strain was represented by two bands on the DGGE gel (lane F) (Fig. 3), indicating that two copies of the 16S rDNA gene were present. The first band was sequenced and showed 98% sequence similarity with *Lactobacillus mucosae*. This strain was named *L. mucosae* strain EPI2. The fact that this strain is member of the *Lactobacillus* genus could be predicted from the fact that the intensity of the bands representing the strain in the DGGE gel increased after incubation in MRS broth, a medium selective for lactic acid bacteria. The last isolate had 99% sequence similarity with *Finegoldia magna* and was named *F. magna* strain EPI3. The nucleotide sequence fragments of the 16S rDNA gene of *E. faecium* strain EPI1, *L. mucosae* strain EPI2 and *F. magna* strain EPI3 have been deposited in GenBank under accession numbers AY672459, AY672460 and AY672461 respectively.

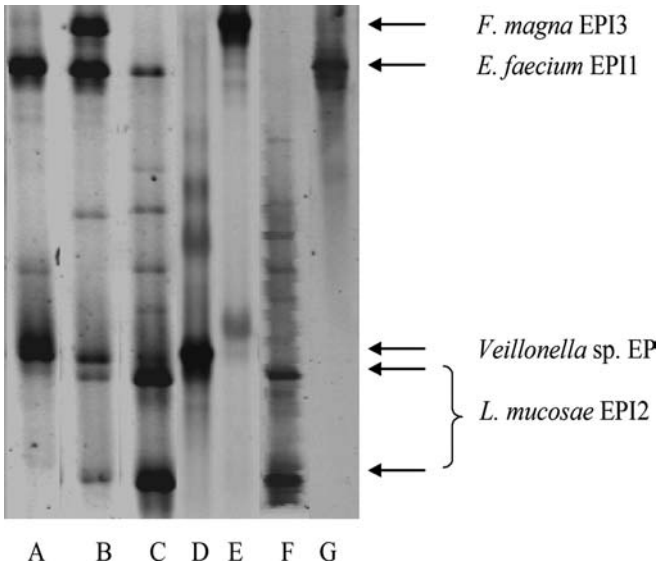

Fig. 3 DGGE gel. *Lane A* EPC4 1 day after inoculation in BHI_a, *lane B* EPC4 after 5 days of incubation in BHI_a, *lane C* EPC4 after 5 days of incubation in MRS, *lane D* clone 1, *lane E* isolate 3, *lane F* isolate 2, *lane G* isolate 1

Further efforts to isolate the species represented by the other band on the DGGE gel were not successful. *E. faecium* EPI1, *L. mucosae* EPI2 and *F. magna* EPI3 did not metabolise daidzein when they were inoculated as single species, nor when they were combined in BHI_a supplemented with daidzein.

For the identification of the species that could not be isolated from the mixed culture through plating on BHI_a, the 16S rDNA of the total culture was cloned. A total of 40 clones were constructed. Two species were predominant in these clones, namely *F. magna* EPI3, and the unidentified species. The latter showed 95% DNA sequence similarity with several *Veillonella* species and was named *Veillonella* sp strain EP. Partial sequence of the 16S rRNA gene was deposited at GenBank under

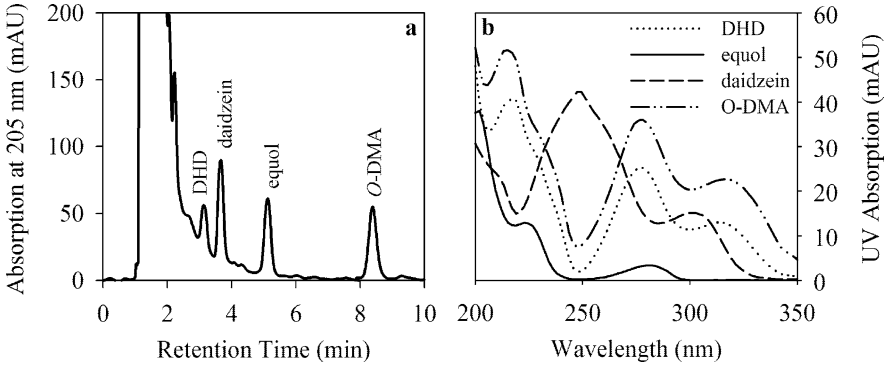

Fig. 2 UV-absorption profile at 205 nm after HPLC elution of a sample containing daidzein and its main intestinal metabolites (**a**). UV-absorption spectra of daidzein, DHD, *O*-DMA and equol (**b**). *AU* Absorbance units

accession number AY672458. The most relevant closely related *Veillonella* species were purchased from a culture collection and were tested for their ability to produce equol in single culture and in combination with the isolated strains, and were found to be unable to produce equol (data not shown).

The mixed culture appeared to be stable. After several transfers to fresh growth medium the culture maintained its ability to convert daidzein into equol. Also, the composition of the culture, as derived from the DGGE pattern (data not shown), did not shift. This equol-producing mixed culture, consisting of four associated bacterial species, was named EPC4.

Influence of environmental conditions on equol production by EPC4

The concentrations of daidzein, equol and DHD during 6 days of incubation under different conditions are presented graphically in Fig. 4. Hydrogen had a major impact on daidzein metabolism. In the flasks where the headspace was filled with hydrogen gas, the growth media were depleted of daidzein after 48 h of incubation in all of the four batches (Fig. 4a). At that point equol concentrations (Fig. 4c) were 36.0 ± 1.7, 42.0 ± 1.8, 38.3 ± 2.1 and 46.8 ± 3.4 μM respectively, for control-H_2, AC-H_2, PR-H_2 and BT-H_2. During further fermentation, a significant ($P < 0.05$) increase in equol concentration within one incubation was only observed in control-H_2. There were no significant differences among the daidzein concentrations within the hydrogen gas group over the whole incubation period. After 6 days, the equol concentrations of control-H_2 and BT-H_2 (53.8 ± 3.4 μmol/l) were significantly higher ($P < 0.05$) than AC-H_2 and PR-H_2 (46.2 ± 0.6 and 41.5 ± 2.5 μmol/l respectively).

The cultures that were incubated under nitrogen gas metabolised daidzein less efficiently. The rate of daidzein metabolism was dependent on the supplementation. Daidzein disappeared completely after 6 days of incubation only in culture PR. Equol concentrations after 6 days were 1.1 ± 1, 57.0 ± 1.1 and 17.0 ± 1.4 μmol/l for control, PR and BT, respectively. No equol was detected in AC.

The concentrations of DHD (Fig. 4b) were variable during this experiment and did not show a clear trend as did daidzein and equol. The sum of the concentrations of daidzein, equol and DHD at the end of the experiment for control, AC, PR, BT, control-H_2, AC-H_2, PR-H_2 and BT-H_2 was respectively 46.4 ± 1.3, 48.0 ± 0.8, 68.0 ± 1.1, 79.6 ± 0.3, 63.7 ± 0.4, 85.6 ± 0.7, 72.3 ± 2.1 and 76.8 ± 2.3% of the originally added concentration of daidzein.

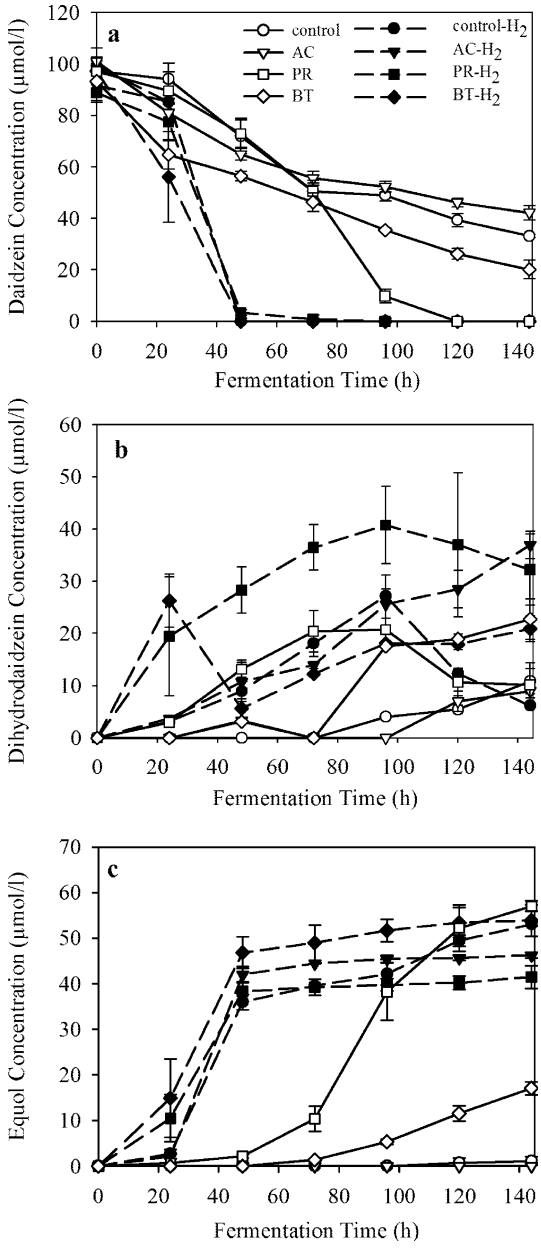

Fig. 4 Daidzein (**a**), DHD (**b**) and equol (**c**) concentrations during incubation of EPC4 in BHI_a at 37°C under different conditions over 6 days. Data are presented as mean ± SD (*error bars*) ($n = 3$)

Influence of the presence of Pd^0 on equol production

In the presence of Pd^0, no equol was produced by EPC4 until 2 weeks after inoculation (Fig. 5). In the absence of the Pd^0 daidzein was transformed into equol. Plate counts showed that no differences in the total numbers of anaerobic bacteria occurred between the cultures where Pd^0 was added or not.

Influence of FOS on equol production

Addition of FOS to the medium inhibited equol production by EPC4, as can be seen from Table 2. Although the daidzein concentration decreased to 38 ± 2 μmol/l, none of the metabolites DHD, *O*-DMA or equol could be detected.

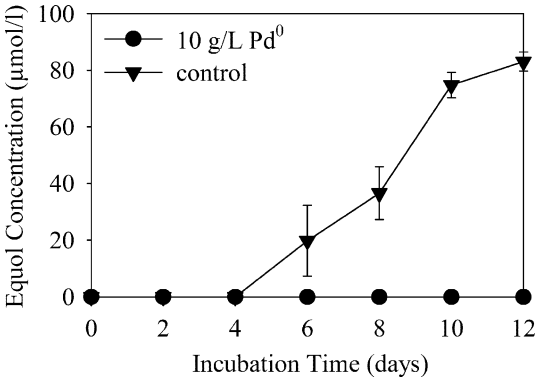

Fig. 5 Concentration of equol during incubation at 37°C with the EPC4 in BHI_a supplemented with 100 μmol daidzein/l under N_2 in presence and absence of 10 g Pd^0 /l. Data presented as mean ± SD ($n = 3$)

Supplementation of EPC4 with a non-equol-producing faecal culture

In Table 2 the results of supplementing EPC4 with faecal cultures originating from non-equol-producing individuals are presented (only for 0, 1 and 50%(v/v) EPC4). Equol was produced in both samples 1 and 2 when EPC4 comprised only 1%(v/v) of the mixture. As the proportion of EPC4 in the total mixture was increased, equol production appeared more rapidly. For sample 1, the final level of equol after 14 days of incubation was comparable for all the different proportions of EPC4 added (data not shown). When FOS were added to the culture medium, no equol was formed. For sample 2, which contained bacteria able to transform daidzein into *O*-DMA, the final level of equol was dependent on the proportion of EPC4 at the beginning of the incubation. After 14 days of incubation the final concentration of equol was 53 ± 4 μmol/l when 1%(v/v) EPC4 was added; 60 ± 5 μmol/l when 5%(v/v) was added; 61 ± 4, 77 ± 3 and 81 ± 2 μmol/l when 25, 50 or 75%(v/v) was added respectively, and finally, when EPC4 was incubated in pure culture, the concentration was 87 ± 8 μmol/l. As the production of equol was increased, the amount of *O*-DMA formed decreased. It should be noted that the rate, not the total amount, of equol production was higher when the EPC4 was mixed with any faecal culture than as 100% EPC4. Again, addition of FOS altered daidzein metabolism by the bacteria. No equol could be detected, and the formation of *O*-DMA was almost completely inhibited.

Discussion

In this study, we have isolated a stable mixed microbial culture, capable of transforming daidzein into equol, from a human faecal sample and examined the influence of some environmental conditions in the colon on equol production. We have also achieved the production of equol upon inoculation of the mixed culture into faecal cultures which originally did not produce equol.

The metabolism of the soy isoflavonoid daidzein by the intestinal microbiota is an important issue with respect to the bioavailability and bioactivity of this phytoestrogen (Rowland et al. 2003). The great interin-

Table 2 Concentrations of daidzein (*Da*), equol (*Eq*) and *O*-DMA (μmol/l) after incubating a mixture of EPC4 and faecal cultures of samples 1 and 2 in different proportions with or without the addition of FOS (10 g/l). Data are presented asthe mean ± SD. *ND* Not detected. No *O*-DMA was detected during incubation of 100% EPC4 or mixtures containing sample 1

EPC4%	Time(day)	Sample 1		Sample 2			FOS				
							Sample 1		Sample 2		
		Da	Eq	Da	Eq	O-DMA	Da	Eq	Da	Eq	*O*-DMA
0	0	100 ± 7	ND	102 ± 8	ND	ND	99 ± 6	ND	100 ± 5	ND	ND
	2	97 ± 5	ND	34 ± 6	ND	34 ± 5	98 ± 7	ND	93 ± 7	ND	2 ± 1
	4	68 ± 6	ND	6 ± 3	ND	47 ± 3	97 ± 5	ND	59 ± 5	ND	3 ± 1
	6	46 ± 3	ND	4 ± 2	ND	47 ± 4	92 ± 4	ND	50 ± 4	ND	3 ± 1
	8	43 ± 4	ND	ND	ND	49 ± 3	53 ± 7	ND	45 ± 3	ND	3 ± 1
1	0	95 ± 5	ND	100 ± 6	ND	ND	99 ± 5	ND	98 ± 6	ND	ND
	2	28 ± 5	25 ± 5	ND	2 ± 2	28 ± 3	96 ± 4	ND	89 ± 5	ND	2 ± 1
	4	2 ± 1	37 ± 6	ND	32 ± 6	28 ± 5	84 ± 6	ND	61 ± 4	ND	2 ± 1
	6	1 ± 0	76 ± 4	ND	39 ± 4	28 ± 4	58 ± 4	ND	55 ± 5	ND	3 ± 2
	8	ND	89 ± 4	ND	53 ± 4	27 ± 3	51 ± 5	ND	50 ± 5	ND	3 ± 1
50	0	93 ± 5	ND	102 ± 5	ND	ND	103 ± 6	ND	99 ± 4	ND	ND
	2	ND	85 ± 6	ND	80 ± 6	5 ± 2	100 ± 6	ND	96 ± 5	ND	1 ± 1
	4	ND	89 ± 4	ND	82 ± 4	6 ± 2	91 ± 4	ND	94 ± 5	ND	1 ± 1
	6	ND	90 ± 3	ND	81 ± 3	6 ± 1	78 ± 5	ND	89 ± 3	ND	2 ± 1
	8	ND	90 ± 4	ND	79 ± 4	6 ± 1	67 ± 4	ND	82 ± 5	ND	3 ± 1
100	0	95 ± 2	ND				97 ± 1	ND			
	2	75 ± 6	ND				79 ± 13	ND			
	4	47 ± 3	ND				52 ± 1	ND			
	6	37 ± 3	18 ± 11				42 ± 5	ND			
	8	15 ± 6	33 ± 8				38 ± 2	ND			

dividual variation in daidzein metabolism observed in in vivo studies with humans has been attributed to variation in the metabolic activity and composition of the intestinal microbiota (Xu et al. 1995; Rafii et al. 2003; Atkinson et al. 2004). The metabolite equol, which is exclusively formed by intestinal bacteria, has attracted considerable attention. The fact that only ~35% of humans possess a microbiota capable of producing this metabolite has been suggested as an explanation for the conflicting results in dietary intervention studies with humans (Setchell et al. 2002). Hence, there is a major interest in understanding the microbiological processes involved in this biotransformation, in order to find ways to modulate this activity through the diet. Our approach was (1) to attempt the isolation of bacteria producing equol, (2) to assess the influence of environmental conditions in the colon on equol production by the bacteria isolated, and (3) to study their activity in a non-equol-producing faecal culture. Results obtained in this study will contribute to a further understanding of in vivo equol production in humans.

One of the faecal samples of the four volunteers examined in this study produced equol in vitro. This is in agreement with the ~35% rate of equol production observed in human studies. Clinical studies report the presence of the other intestinal metabolite of daidzein, *O*-DMA, in most humans, independently of their equol-producing status (Lampe et al. 2001). In this study, only one sample produced *O*-DMA in vitro and this did not coincide with equol production. A similar observation was made in a recent study by Rafii et al. (2003), where only one faecal culture out of six produced *O*-DMA after one sampling round.

We attempted to isolate a single strain capable of transforming daidzein into equol from the faecal sample that produced equol. We could isolate a stable mixed microbial culture producing equol, consisting of four associated bacteria which were identified as *E. faecium* strain EPI1, *L. mucosae* strain EPI2, *F. magna* strain EPI3 and an as yet undescribed species related to *Veillonella* sp. Only the first three could be brought in culture in BHI_a individually, but they did not produce equol or DHD from daidzein. The *Veillonella* sp related bacterium could not be isolated through conventional plating techniques. Therefore, we tested several strains obtained from a culture collection closely related to the bacterium found in the mixed culture. However, none of the tested strains was able to produce equol. When the three isolated bacteria and the purchased *Veillonella* sp strains were combined, again no equol production was observed. This could indicate that substituting the poorly identified species by the different *Veillonella* strains did not mimic the equol-producing mixed culture, or that one or more species, present in very small numbers and whose presence did not result in a DGGE band or a 16S rRNA clone because of bias in PCR amplification, contributed to equol production. The latter is, in view of the sensitivity of the PCR detection which normally reveals species present above 0.5% of the total population, considered very unlikely. From the results obtained so far in this study, it cannot be concluded whether production of DHD or equol by EPC4 is the result of the action of one single species or the interaction of more. Bacterial species that have been reported to transform daidzein into DHD as single strain (Hur et al. 2000) were not found to be present in EPC4. Further efforts to unravel the mechanism behind equol production in this mixed culture are needed.

Supplementing two faecal cultures of non-equol-producing bacteria with EPC4 resulted in equol production in both cultures. This indicates that the bacteria present in EPC4 were able to maintain their activity in the microbial community of the faecal sample, although it cannot be excluded that the bacteria that produce equol, possibly being present in small amounts in the faecal culture, were stimulated by supplementation with EPC4. Interestingly, *O*-DMA formation was suppressed in a dose-dependant way in sample 2 upon supplementation with EPC4. This suggests that equol and *O*-DMA are formed by different bacteria, which has already been reported by others (Hur et al. 2002). Apparently, these bacteria are able to co-exist, as *O*-DMA and equol were both produced when EPC4 was added to sample 2. Also, in humans, the formation of equol does not exclude *O*-DMA production (Lampe 2003). We observed a direct competition between these microorganisms. A similar observation was made by Kelly et al. (1995), who found an inverse relationship between the amount of equol and *O*-DMA in humans after a soy challenge. Equol production in the non-producing samples was observed when as little as 1(v/v)% of EPC4 was added, which indicates a potential use for this culture as a probiotic for the stimulation of equol production.

In the experiments we performed (Fig. 3), the mass balance of daidzein could not always be equilibrated, even though the extraction efficiency was satisfying. When BHI_a supplemented with daidzein was incubated at 37°C without inoculation of bacteria, the daidzein concentration remained at the initial level for several weeks under the conditions used in the experiment (data not shown). However, in the treatments where no equol was formed (control and AC), less than 50% of the daidzein initially added could be recovered as daidzein and metabolites. This indicates that biotransformation products other than DHD, *O*-DMA and equol may be produced by the intestinal microbiota, or that physiochemical interactions with components of the culture media or bacteria prevent the efficient extraction of these phytochemicals. The persistence of equol after formation confirms the earlier findings that equol, once formed, is in general metabolically stable (Setchell et al. 2002).

Hydrogen gas and short chain fatty acids are present in high concentrations in the gut as they are the major metabolic products of the fermentation of carbohydrates by the gut microbiota (Macfarlane and Macfarlane 2003). Hydrogen in particular, but also propionate and butyrate influenced equol production in a positive way.

The importance of H_2 was shown when the mixed culture was incubated in the presence of Pd^0. Metallic palladium can absorb 900 times its own volume of H_2 (Zumdahl 1998), but in the ionic form it is toxic towards bacteria. The non-ionic form is insoluble and therefore considerably less toxic (Liu et al. 1979), as could be observed from the similar bacterial counts in the control experiment. By adding the Pd^0, microbially produced hydrogen gas was immobilised and no longer available to the bacteria. Under these conditions no equol was produced by the bacteria, indicating an important role for hydrogen gas in the mechanism of equol production. The influence of the SCFAs could be explained also in relationship to H_2. It is known that acetate can be used by bacteria in the human colon to produce propionic and butyric acid, thus consuming H_2 (Duncan et al. 2002). It is possible that the inhibitory effect of acetate on equol production was due to hydrogen consumption by bacteria producing propionate or butyrate. Conversely, the presence of high concentrations propionate or butyrate could prevent this hydrogen consumption through a feedback mechanism. Regardless, the microbial interactions in the colon are exceedingly complex and to date are not well understood (Dolfing and Gottschal 1997).

By incubating EPC4 in the presence of hydrogen gas and SCFAs, we tried to simulate a diet rich in carbohydrates. Based on the data obtained, one could assume that a diet rich in carbohydrates may stimulate equol production in an individual harbouring an intestinal microbiota which contains equol-producing bacterial species. This has already been suggested by others (Rowland et al. 2000; Lampe et al. 2001). To further explore this hypothesis, FOS were added to the growth medium in which EPC4 was grown. FOS are a mixture of indigestible and fermentable β-D-fructans with variable degree of polymerisation (DP), in this case the DP was 10. They are believed to typically alter the composition of the human colon microbiota towards a predominance of bifidobacteria (Gibson and Roberfroid 1995; Kleessen et al. 1997; Fooks et al. 1999; Hopkins and Macfarlane 2003). Addition of FOS had a negative influence on equol production by EPC4. Furthermore, when FOS were added to faecal cultures supplemented with EPC4, an inhibitory effect on equol production was observed. Metabolism of FOS by intestinal bacteria results in a large release of hydrogen (Cummings et al. 2001), the latter stimulating equol production according to our results (Fig. 4). However, the findings from the experiments with FOS in this study are contradictory to the hypothesis that FOS enhance equol production in this way. Allthough no bifidobacteria were detected in EPC4 it is probable that the presence of FOS altered the culture composition and fermentation pattern. These changes can cause a decrease in the equol-producing microbial population and simultaneously a shift in hydrogen utilisation. As a result daidzein is no longer transformed to DHD or equol. A decrease in equol production was also reported by Zafar et al. (2004) when rats were fed inulin and isoflavones, which is composed of FOS with a different DP, while others found an increase in equol production when rats were fed FOS and isoflavones (Ohta et al. 2002). Several studies report an increase in daidzein bioavailability upon ingestion of a diet rich in carbohydrates and dietary fibres (Tew et al. 1996; Lampe 2003; Steer et al. 2003). This is in concordance with our findings, where FOS inhibited the formation of equol and DHD. As daidzein is less subjected to bacterial metabolism in vivo, it is more likely to be absorbed resulting in an increased bioavailability. Equol production appeared to be faster when EPC4 was added to the fecal cultures than in EPC4 alone. This effect is very likely to be due to the high hydrogen production by the microbiota from the faecal samples, where a greater numbers and diversity of hydrogen producing bacteria are present.

Hydrogen gas is an important intermediate in the intestinal ecosystem and is formed by a variety of hydrolytic and saccharolytic bacteria as a mean of disposing of reducing equivalents from the anaerobic environment of the colon. However, a high partial pressure of H_2 inhibits fermentation efficiency in anaerobic ecosystems (Wolin and Miller 1983; Christl et al. 1992). In humans, part of the H_2 formed by colonic bacteria is excreted in breath and flatus, but the most important way of hydrogen disposal is interspecies hydrogen transfer (Christl et al. 1992). In the colon in vivo there are three main groups of hydrogen consuming bacteria, i.e. sulfate reducing bacteria, methanogens and reductive acetogens. There is competition between these groups for the common substrate, hydrogen, resulting in the dominance of one group over the other two (Gibson et al. 1990; Florin et al. 2000). So far, no relationship between equol production and the different modes of hydrogen consumption has been discovered. However, the results from this study indicate a strong relationship between hydrogen consumption and equol production. Probably, hydrogen gas acts as an electron donor in the biotransformation reaction from daidzein to equol. If the electron balance of a reaction with hydrogen gas is equilibrated, three molecules of H_2 are required for the reduction of one molecule daidzein to equol. This reduction could be an alternative strategy for certain intestinal bacteria to deposit reducing equivalents. The interactions between equol-producing bacterial cultures and methanogens, sulfate reducing bacteria and reductive acetogens are interesting topics for further research.

In conclusion, a mixed bacterial culture, originating from a human faecal sample, which converts daidzein into equol in vitro has been isolated and described in this study. The culture could be restricted to four dominant bacterial strains, but we have not yet identified the bacterium responsible for equol production. Equol production was stimulated to a large extent by hydrogen gas, probably acting as electron donor in the biotransformation. Increased equol production was also found in the presence of propionate and butyrate, suggesting that a diet rich in carbohydrates stimulates equol pro-

duction. However, it was found that adding FOS was inhibitory for equol production. This observation requires further investigation. Furthermore, we showed that the addition of this mixed culture to a faecal culture from a non-equol-producing individual stimulated equol production. This is a first indication that equol-producing bacteria have a potential use as a probiotic for the in vivo stimulation of equol production.

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