Anaerobe 25 (2014) 47–52

Contents lists available at ScienceDirect

Anaerobe

journal homepage: www.elsevier.com/locate/anaerobe

Clinical microbiology

Lactulose promotes equol production and changes the microbial community during *in vitro* fermentation of daidzein by fecal inocula of sows

Weijiang Zheng [a], Yanjun Hou [a], Yong Su [a], Wen Yao [a,b,*]

[a] College of Animal Science and Technology, Nanjing Agricultural University, Nanjing, Jiangsu 210095, China
[b] Key Lab of Animal Physiology and Biochemistry, Ministry of Agriculture, Nanjing, Jiangsu 210095, China

ARTICLE INFO

Article history:
Received 22 April 2013
Received in revised form
21 November 2013
Accepted 25 November 2013
Available online 4 December 2013

Keywords:
Lactulose
Hydrogen-producing prebiotic
Hydrogen-utilizing bacteria
Equol
Sows' feces

ABSTRACT

Equol has higher biological effects than other isoflavones. However, only about 30–50% of humans possess a microbiota capable of producing equol from dietary daidzein. In recent years, interest has grown in dietary applications to improve equol production in human and other animals. In this study, lactulose was used as a potential equol-promoting prebiotic *in vitro*. The effect of lactulose on transformation of daidzein into equol by sows' fecal microbiota was investigated. Results showed that lactulose treatment improved bacteria growth parameters, changing the kinetics of fermentation *in vitro*. Lactulose significantly increased total gas production, *T*1/2, *T*max, and *R*max. Furthermore, lactulose altered the microflora composition, increased equol production associated with a reduction in the population of methanogen and increased the sulfate-reducing bacteria population during 24 h of incubation. Here, we report for the first time that in a certain condition (sealing or high pressure), via a dihydrodaidzein (DHD) pathway equol might be able to reform to daidzein by further metabolism using lactulose as a substrate. This study proposes that "hydrogen-producing prebiotic" might be a novel way to promote equol production *in vivo* or *in vitro*.

1. Introduction

Daidzein, a major soybean isoflavone, is metabolized to O-desmethylangolensin and equol in the gastrointestinal tract by gut microflora [1]. Equol has attracted a lot of attention since postulation of the equol hypothesis, contending greater efficacy of soy products in so-called equol producers [2]. It has been well established that equol has a higher oestrogenicity [3,4] than its precursor daidzein, is a more potent antioxidant [5], and possesses anti-inflammatory properties [6]. Furthermore, equol has a higher effective free fraction circulating in human serum and a slower plasma clearance compared to daidzein [2]. Although there is conflicting evidence regarding potential health benefits associated with ability to produce equol, there is growing interest in dietary applications that can enhance equol in humans and other animals. However, the profile of the microflora is a determining factor for the metabolites formed, and is variable among individuals [7,8]. Recent studies have shown that hydrogen gas plays an important role in the mechanism of equol formation [9,10]. Several studies have examined the effect of both prebiotics and probiotics on equol production in an attempt to promote the beneficial effects of isoflavones [11–15].

The hypothesis of this study is that equol production can be improved by adding hydrogen-producing prebiotic *in vivo*/*in vitro*. To our knowledge, studies in this area are rare. Lactulose, a disaccharide formed from one molecule each of the simple sugars fructose and galactose, cannot be digested and absorbed by the body but can be digested by bacteria colonizing the gastrointestinal tract, especially the colon. One of the main byproducts of its digestion is H_2. Oral administration of lactulose significantly increases H_2 production [16]. Based on observations and experiments, we hypothesized that lactulose may be a novel equol-promoting prebiotic. Changing the microbiota profile and increasing microbial-derived H_2 may significantly increase equol production, improving the biological activity of

* Corresponding author. College of Animal Science and Technology, Nanjing Agricultural University, Nanjing, Jiangsu 210095, China. Tel.: +86 025 84395523; fax: +86 025 84395314.
E-mail addresses: zwj0393@163.com (W. Zheng), hyjnanjing@hotmail.com (Y. Hou), suney7912@yahoo.com (Y. Su), yaowen67jp@njau.edu.cn (W. Yao).

1075-9964/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.anaerobe.2013.11.011

soy isoflavones in humans and animals. This study examined the effect of lactulose on equol-producing capacity of faecal microbiota of sows *in vitro*, via high-performance liquid chromatography coupled with mass spectrometry (LC/MS), polymerase chain reaction-denatured gradient gel electrophoresis (PCR-DGGE), and quantitative real-time polymerase chain reaction (RT-PCR) to characterize the composition of corresponding microflora and its management by lactulose.

2. Materials and methods

2.1. Experimental design

Fecal samples from six large white gestating sows with fecal equol levels above 15.0 μg/g (wet weight) were used as inocula. Three grams of each freshly voided fecal sample was homogenized and suspended in 180 mL of an autoclaved phosphate buffered saline (PBS) buffer and then filtered through a double layer of sterile cheesecloth. Then, 5 mL of the resulting mixture was added to serum bottles containing 45 mL of autoclaved brain heart infusion (BHI) supplemented with 0.5 g L-cysteine/L and with or without 200 μmoL/L daidzein.

Based on substrates in media, the study consisted of four groups including blank (*C*), daidzein (200 μmoL/L) (*D*), lactulose (10 g/L) (*L*), and lactulose (10 g/L) with daidzein (200 μmoL/L) (*D* + *L*). During incubation at 37 °C for 72 h, gas production was measured using the pressure transducer technique of Theodorou et al. [17]. Daily samples were taken for HPLC analysis and DNA isolation. This *in vitro* experiment was repeated twice.

2.2. Analytical methods

2.2.1. Extraction and quantification of daidzein and equol

Equol concentration was determined according to the description of Decroos et al. [9]. One of culture fluid was extracted with diethyl ether and ether fractions in the supernatant were combined, evaporated to dryness, dissolved in 500 μl of 80% (v/v) ethanol, and stored at −20 °C until analysis.

Culture extracts and standard daidzein and equol purchased from Sigma–Aldrich were analyzed by high-performance liquid chromatography (HPLC: Thermo Finnigan™ Surveyor and autosampler, USA) coupled to an ion trap mass spectrometer (Thermo Finnigan™ LCQ Deca XP Plus, USA) fitted with an electrospray source. This LC/MS platform including a Finnigan Surveyor MS Pump Plus, Thermo Scientific Surveyor Autosampler, Finnigan Surveyor PDA Plus Detector and Thermo Fisher Xcalibur™ software package (version 1.4 SR1). Qualitative analysis was based on retention time, and quantitation was carried out with intensity of the external standard, LC/MS was carried out the following conditions.

2.2.1.1. Chromatographic conditions. A 20 μl sample was injected and separated over a YMC-Pack Pro C_{18} (250 mm × 3 mm, particle size 5 μm, Japan). The temperature was set at 30 °C and the flow rate was maintained at 300 μL/min. Elution was isocratic with a mobile phase consisting of acetonitrile: methanol: water (20:35:45). Equol was detected at 205 nm and daidzein at 260 nm. The concentration of equol was calculated following the equation $Y = 85653.6 + 1.23123e{+}006{*}X$ (*Y*: HPLC peak area; *X*: concentration of equol), and the daidzein following the equation $Y = 36529.5 + 844385{*}X$ (*Y*: HPLC peak area; *X*: concentration of daidzein).

2.2.1.2. Mass spectrometry. The instrument was operated in the ESI-negative monitoring the ions with $(m/z)^-$ 253 daidzein, $(m/z)^-$ 255 dihydrodaidzein (DHD), $(m/z)^-$ 241 equol. The best results were obtained with a spray voltage of 4.5 kV, capillary temperature of 275 °C and sheath gas flow rate of 20 units.

2.2.2. DNA isolation and PCR amplification

DNA of the cultures was extracted according to the method of Zoetendal et al. [18]. Table S1 lists all PCR primers used in this study. Primers U968-GC and L1401 [19] were used to amplify the V6–V8 regions of the bacterial 16S rRNA gene. PCR products were separated by denatured gradient gel electrophoresis (DGGE) [20], using a Dcode™ system (Bio-Rad, Hercules, CA) in 8% polyacrylamide gels with denaturing gradient of 38–55%. Gels were stained with $AgNO_3$ [21], and scanned by a GS-800 Calibrated Densitometer (Bio-Rad) for analysis by GelCompare II 5.0 (Applied Maths, Kortrijk, Belgium).

2.2.3. Cloning of PCR-amplified products and sequence analysis

Almost full-length 16S rRNA genes from a DNA sample from the *D* + *L* group incubated for 72 h was amplified by PCR amplification using primers 8F and 1510R [22] using the Taq DNA polymerase kit (Takara, Dalian, China). Purified PCR products were cloned in *Escherichia coli* JM109 using the pGEM-T vector system (Invitrogen, Shanghai, China). Clonal colonies of ampicillin-resistant transformants were picked after overnight growth, transferred to Luria broth medium, and incubated at 37 °C overnight. One hundred μL of the above cultures were centrifuged and suspended in 100 μL TE buffer. Then, the solutions were boiled for 5 min to lyse the cells. The cell lysates were used as templates in PCR reactions using pGEM-T specific primers Sp6 and T7 to check the size of the DNA inserts. The plasmids with appropriately sized inserts were used to amplify the V6–V8 regions by PCR as described above. Then, the amplicons were compared with 72 h in the *D* + *L* group DNA-derived PCR products from the same samples in DGGE profiles. Amplicons corresponding to interesting bands on the same sample in DGGE profiles were selected and sent to sequencing (Invitrogen, Shanghai). The assembled partial 16S rRNA gene sequences were compared with sequences from the GenBank database.

2.2.4. Real-time PCR assays for the quantification of total bacteria, methanogen producing bacteria and sulfate-reducing bacteria

Methanogen (mcrA) [23] and subunit A of the adenosine-5′-phosphosulfate (APS) reductase gene [24] from cultures were amplified with primers shown in Table S1. Purified PCR products were cloned in *E. coli* JM109 using the pGEM-T vector system (Invitrogen, Shanghai, China). Plasmid DNA with correct size inserts was extracted and used for constructing standard curves. The standard curves were generated using triplicate ten-fold dilutions of plasmid DNA. For all real-time PCR assays, there was a linear relationship between the log of plasmid DNA copy number and the calculated C_T value across the specified concentration range ($R^2 = 0.99$ in all cases) (data not shown). Real time PCR was carried out using an ABI7300 real-time PCR machine (Applied Biosystems, Foster City, CA, USA) in a total volume of 20 μL containing 10 μL 2 × SYBR Premix Ex Taq (Takara Dalian China), 2 μL DNA (1:9 diluted with PCR-grade H_2O); 0.4 μL each of 10 μmoL/L forward and reverse primers, 0.4 μl ROX and 4.2 μL PCR-grade water. The program was set at 95 °C for 10 s, followed by 40 cycles of 95 °C for 5 s, and 60 °C for 31 s. Melting curve analysis of amplification products was performed at the end of each PCR reaction to confirm that only one PCR product was amplified and detected.

2.2.5. Nucleotide sequence accession numbers

Twelve bacterial 16S rRNA gene sequences from the 72-h sample in *D* + *L* group were obtained, and were deposited in the GeneBank database under accession numbers: KC484759,