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# Equol Inhibits Growth and Spore Formation of *Clostridioides difficile*

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Running Head: Equol Inhibits Spore Formation of bacteria

***Abstract***

Aims:

Equol is a non-steroidal estrogen of the isoflavone class. We investigated the antibacterial ability of equol with respect to the growth rate, toxin-production, and spore-forming abilities of *Clostridioides difficile* BI/027/NAP1.

Methods and Results

Isoflavones, or female hormones, were added to bacterial culture, which was grown at 35 °C. The absorbance of the culture was measured at various time-points for evaluating the growth inhibition. The toxin levels in the media and morphological changes were also assessed. To evaluate the influence of equol on the sporulation of *C. difficile*, cells were collected at various time-points from the equol-supplemented culture and the number of spores was counted. Our results show that equol inhibits bacterial growth in a concentration-dependent manner. However, it does not inhibit the production of toxin by *C. difficile*. Other isoflavones and female hormones did not inhibit the *C. difficile* growth. At the 14th day, approximately 600 spores were present in the control medium and only six were seen in the equol-containing medium.

Conclusion

Our results suggest that equol may directly inhibit the *C. difficile* growth in a concentration-dependent manner and spore formation.

Significance and Impact of the Study

This is the first report on the antimicrobial ability of equol.

Key words

equol, isoflavones, *C. difficile*, antibacterial ability, infection, spore formation

**Introduction**

*Clostridioides difficile* (formerly *Clostridium difficile*) (Lawson *et al.* 2016; Oren *et al.* 2016) is a gram-positive, spore-forming, toxin-producing, anaerobic bacterium, which has established itself as a leading cause of nosocomial antibiotic-associated diarrhea in developed countries (Rupnik *et al.* 2009; Chakra *et al.* 2016). It is found widely in the mammalian gastrointestinal tract and can cause toxin-mediated *C. difficile* infections (CDI) that range from mild diarrhea to pseudomembranous colitis and potential death. Although the standard antimicrobial treatment for CDI is effective, disease recurrence after the initial treatment can still reach up to 15-35% (Leffler *et al.* 2015). One of the reasons for the high recurrence rate is that *C. difficile* spores could still be present within the patients gut and they germinate to the vegetative form after completion or discontinuation of antibiotic treatment (Cornely *et al.* 2012).

Soybean isoflavones have structures similar to estrogen and bind to estrogen receptors, suggesting that they may exhibit an estrogenic action in various tissues (Breinholt *et al.* 1998; Schmitt *et al.*

2001) and therefore might prevent postmenopausal disorders such as osteoporosis (Taku *et al.* 2010). Equol is a metabolite of iso daidzein, produced by bacteria in the distal intestine and colon. Intestinal bacteria have an essential role in daidzein metabolism, and specific equol-producing bacteria have been reported (Atkinson *et al.* 2005). Resistant starch (RS) is a type of dietary fiber that includes all starch and starch degradation products that are not absorbed in the small intestine of healthy humans (Topping *et al.* 2003). RS is fermented to a large extent by the microbiota in the colon, resulting in the production of short-chain fatty acids, which lower the pH in the colon (Topping *et al.* 2001). Several studies have characterized the potential of RS to induce alterations in the composition of the gut microbiota and have reported increases in *Bifidobacteria*, *Lactobacillus* spp. and *Bacteroides* (Wang *et al.* 2002; Conlon *et al.* 2009; Tousen *et al.* 2011; Zhang *et al.* 2013). The combination of isoflavones and RS prevented an ovariectomy-induced decrease in trabecular bone mass and bone strength in the distal femur by modulating the enteric environment. This modulation included an increase in the abundance of *Bifidobacerium* spp. and equol production, and regulating inflammation-related genes in the bone marrow (Tousen *et al.* 2011). The ability to produce equol depends on the presence of certain intestinal microbiota, that the enteric environment can affect equol production (Yuan *et al.* 2007). Certain *Bifidobacterium* and *Lactobacillus* species have been suggested to have an important role in the metabolism of daidzein to equol (Xu *et al.* 1995; Tsangalis *et al.* 2002; Topping *et al.* 2003; Decroos *et al.* 2005;). A previous study reported that the composition of caecal intestinal microbiota in mice that were fed combination food of RS and isoflavones was

altered, with an increase in *Lactobacillus* spp. and a concomitant decrease in the *Clostridium* spp. (Tousen *et al.* 2016).

In this study, we investigated the antibacterial ability of equol on *C. difficile* growth, toxin production, and sporulation with an aim to prevent CDI outbreaks, especially in Asia.

## Materials and Methods

### Bacterial strains and growth conditions

A clinical isolate of *Clostridioides difficile* PCR ribotype BI/NAP1/027 (*C. difficile* 027) was kindly provided by Thomas V. Riley, Division of Microbiology and Infectious Diseases, Path West Laboratory Medicine, Perth, Australia. The other three *C. difficile* strains, TUM12745 (PCR ribotype QX104), TUM12806 (UK131), TUM12855 (UK131), and *Bacteroides fragilis* used in this study were clinical isolates stocked in our laboratory. *Escherichia coli* and *Clostridium perfrigens*, were purchased from American Type Culture collection, ATCC35210, and ATCC25285, respectively. All *C. difficile* strains and *C. perfringens* were grown on brain heart infusion media (HBI). Culture were grown at 35°C under anaerobic conditions using Anaero Pack-Anaero (MITSUBISHI GAS CHEMICAL, Tokyo, Japan), a gas mixture containing 10% $CO_2$. *E. coli* was grown on LB medium at 35°C under the aerobic condition. *B. fragilis* was grown on GAM (Nissui Pharmaceutical co., Tokyo, Japan) at 35°C under anaerobic condition.

*C. difficile* and *C. perfringens* were inoculated from glycerol stocks for 48 h (*E. coli*: 24 h, *B. fragiris*: 72 h) onto HBI plates containing taurocholate (0.1% w/v, 1.9 mmol $l^{-1}$) (nutrition medium and GAM were used for *E. coli* and *B. fragilis*, respectively). Single colony from the plates were used to inoculate liquid BHI (or LB, GAM), and the cultures were grown early stationary phase and then diluted with BHI (or LB, GAM) until photometer optimal density at 600 nm ($OD_{600}$) was 0.5, corresponding to $10^4$~$10^5$ CFU $ml^{-1}$. This diluted medium was used in examination of growth, toxin production and spore formation.

Material

S-equol used in this study was purchased from DAICEL (Osaka, Japan). Other isoflavones, daidzein and dihydrodaidzein, were purchased from Sigma-Aldrich (Missouri, USA). Female hormone, estrogen, estradiol and estriol, were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). Substrates were dissolved in dimethyl sulfoxide (DMSO), and concentrated solution was added to bacterial cultures to maintain 0.4% DMSO concentration in the culture. Culture medium that included only 0.4 % DMSO was used as a control.

Measurement of bacterial growth and level of toxin production

Bacterial suspension (50 µl, adjusted to $10^4$ to $10^5$ CFU $ml^{-1}$) and equol or other hormones adjusted to a final concentration of 50 or 100 µg $ml^{-1}$ were mixed with 5 ml HBI and the bacteria were cultured

under the condition described above. DMSO, at the final concentration of 0.4%, without any hormones was used as a control. The absorbance of the cultures at $OD_{600}$ were measured at various time points using a photometer. Morphology of bacterial cells was observed using OLYMPUS BX50 microscope at a magnification of 1000-fold. At the same time, level of toxin in medium was tested by the rapid enzyme immunoassay cassette, *TOX A/B QUIK CHEK*® test (Abbott, California, USA) following manufacturer's instructions and analyzed line density by CD analyzer 3 (ATTO, Tokyo, Japan).

Spore formation

Equol (or DMSO as control) was added to 5 ml of the diluted culture and incubated at 35°C until spore counting. At various culture time points, cells were fixed on a glass slide and stained by Moeller method (Winckel *et al.* 1877). The sample was observed with the OLYMPUS BX50 microscope at 1000-fold magnification. We counted number of red stained spores per field of view and calculated average of ten such fields.

Real-time polymerase chain reaction

RNA of evaluated *C. difficile* was purified using RNeasy® Mini Kit (Qiagen, Hilden, Germany) and stored at –80°C. Equal amounts of RNA were used for cDNA synthesis, using the High-Capacity cDNA Reverse Transcription Kits (Thermo Fisher Scientific, MA, USA), following manufacturer's

instructions. Real-time PCR was carried out on a 7500 Fast Real-time PCR System (Thermo Fisher Scientific, MA, USA) using the Fast SYBR® Green Master Mix (Thermo Fisher Scientific, MA, USA), following manufacturer's instructions. We examined the expression of toxin A, B, and binary toxin by real-time PCR using tcdA (Lyras *et al.* 2009), tcdB (Lyras *et al.* 2009), cdtA (Merino *et al.* 2014), cdtB (Merino *et al.* 2014), rpoA (Lyras *et al.* 2009), and 16SrRNA (Buffie *et al.* 2015) primers.

**Results**

Equol inhibits growth of *C. difficile*

To investigate the effect of equol on growth rate of *C. difficile* 027, the $OD_{600}$ values were measured at various time points. $OD_{600}$ of control culture without equol was 1.700 after 24 h, while that in presence of equol at 50 μg $ml^{-1}$ and 100 μg $ml^{-1}$ were 0.486 and 0.004, respectively (Fig. 1). Thus, equol inhibits the bacterial growth in a concentration dependent manner. We further evaluated the growth of other *C. difficile* strains. All evaluated strains showed the same growth pattern as *C. difficile* 027 (Fig. 2) in both equol-containing and control media, suggesting that equol can inhibit multiple strains of *C. difficile*.

Equol does not inhibit toxin production by *C. difficile*

*C. difficile* 027 secretes three toxins, clostridial toxin A and B and binary toxin. The toxigenic information of the other *C. difficile* strains examined is shown in Table 1. To investigate the effect of equol on toxin production by *C. difficile* 027, we tested toxin levels in the medium at 18, 42 and 72 h after culturing using Toxin A/B test. Intensity of test line was measured by densitometer (Fig. 3). It was previously shown that the line intensity depends on the toxin concentration in the media by toxin dilution test (data not shown). Toxin A/B was detected from control culture after 18 h and the levels gradually increased up to 72 h. In contrast, the toxins could not be detected in the culture containing 100 μg $ml^{-1}$ equol at 18 h, likely because *C. difficile* 027 did not show any growth under these conditions. However, *C. difficile* 027 began to grow after reaching the stationary phase at 42 h. Subsequently, toxin levels in the medium could be confirmed at this time point. In addition, we evaluated the mRNA expression of the toxin producing genes tcdA, tcdB, cdtA, and cdtB, and found no significant differences between presence and absence of equol (data not shown). Thus, equol does not inhibit toxin production.

Equol inhibits spore formation in *C. difficile*

After 2, 5, 9 and 14 days of culture, we counted the number of spores produced by optical microscope. We found over 20 spores per field of view after 2 days in control medium, while only ~2 spores were found in medium containing 100 μg $ml^{-1}$ equol (Fig. 4A). At the $14^{th}$ day, approximately

600 spores were determined in the control medium and only 6 spores were seen in equol-containing medium. Consistent with these observations, the numbers of long vegetative cells were far more abundant in the equol-containing medium (Fig. 4 B, blue cell) than in the control culture where they could barely be detected (Fig. 4 C).

Effect of Equol on growth of *E. coli*, *C. perflingens*, and *B. fragilis*

To investigate effect of equol on other bacteria, three intestinal bacteria were examined (Fig. 5). *E. coli* showed same growth rate in presence and absence of equol. On the other hand, *C. perfringens* showed slower growth in presence of 100 μg $ml^{-1}$ equol compared with growth in control medium, while *B. fragilis* did not grow in the 100 μg $ml^{-1}$ equol medium. However, we confirmed that *B. fragilis* that did not grow in equol medium maintains its ability to form colonies on GAM agar. Thus, equol does not have a bactericidal effect, but rather possess a strong growth inhibitory effect on some bacteria.

Other is flavones do not inhibit growth of *C. difficile*

Equol is metabolized from daidzein through dihydrodaidzein in the intestinal tract and their molecular structure was similar to each other (Fig. 6). To investigate the effect of these other isoflavones on growth of *C. difficile*, we evaluated the $OD_{600}$, 24 h after adding 50 μg $ml^{-1}$ of daidzein or dihydrodaidzein. The $OD_{600}$ values of either culture were same as that measured for the control

culture. Thus, daidzein and dihydrodaidzein do not inhibit the growth of *C. difficile*. On the other hand, in 100 μg $ml^{-1}$ dihydrodaidzein or 50 μg $ml^{-1}$ equol containing medium, the $OD_{600}$ of *C. difficile* culture was 0.450~0.550. Thus, the end products of the daidzein metabolic pathway seem to be more potent in inhibiting bacteria.

Female hormone does not inhibit growth of *C. difficile*.

It was reported that equol has weak estrogen activity. Therefore, we investigated the female hormones, Estrone, Estrasiol, and Estriol, for potential antibacterial activity. However, all hormones used in this experiment did not display any growth inhibition of *C. difficile*. (Fig. 7)

**Discussion**

In this study, we investigated the effect of equol on *C. difficile*. Our results indicate that equol can inhibit the growth of multiple *C. difficile* strains and some other bacteria. In addition, spore formation in *C. difficile* was suppressed in presence of equol. Inhibition of spore production was reported in fidaxomicin-treated *C. difficile* (Babakhani *et al.* 2012). Fidaxomicin inhibits bacterial RNA polymerase activity at a very early stage of the transcription process that must occur continuously during growth and sporulation for the cell to produce a spore. When fidaxomicin was added to an early stationary phase culture, the number of heat-resistant spores decreased to 0.6-1.2 % of that seen in the control. Equol also showed similar inhibitory activity against spore formation in the

stationary phase. On the other hand, it is reported that rifampin added to the exponential growth phase inhibits spore formation in *Clostridium botulinum* more effectively than when added in the stationary phase. However, in this study, we evaluated the reduction of spore formation using stationary phase cells to avoid influence by a fluctuation of cell number.

Inhibition of bacterial growth by isoflavone has been reported previously (Ulanowska *et al.* 2006). Genistein, one of the isoflavones and metabolite of daidzin or genistin, causes a severe inhibition of *Vibrio harveyi* growth in a clear dose-response manner. Especially, the induction of elongated cells suggested that problems with cell division and/or completion of chromosome replication occurred upon genistein treatment of *V. harveyi*. DNA and RNA syntheses were also strongly inhibited in *V. harveyi* relatively shortly after addition of genistein, suggesting that at least one of these processes may be a target for the antibacterial activity of this isoflavone. Genistein might significantly influence DNA topology, leading to negative effects on DNA replication and transcription of genes (Ulanowska *et al.* 2006). In addition, this isoflavone has intermediate effect on *Bacillus subtilis* and no effect on *Escherichia coli*. Similarly, in our study, *E. coli* growth was not inhibited by equol, although the spore forming *Clostridium* species was inhibited. Furthermore, these equol treated cells exhibited elongated shapes. Thus, there is a possibility that equol influences DNA topology in a similar manner as genistein. Moreover, because both equol and genistein are metabolic products of daigin, a major content of soybean, both metabolites possess similar structure. It is known that the structure can influence the strength of antimicrobial activity (Hummelova *et al.* 2015).

Isoflavones having the most potent antibacterial activity contain hydroxyl groups at $R_3$ and $R_6$ position (Fig. 8) and show strong effect against *Staphylococcus aureus* strains, one of the intestinal and gram-positive bacteria, similar to *C. difficile*. Equol, which showed antibacterial activity in this study, also possesses a hydroxyl group at both positions, which could be a characteristic feature for the antibacterial activity. However, the underlying molecular and genetic mechanism of the antibacterial activity remains to be elucidated.

Equol structure is very similar to that of the female hormone estrogen and has weak estrogenic activity (Breinholt *et al.* 1998; Schmitt *et al.* 2001). The estrogenic activities of isoflavones depend on the binding affinity to the estrogen receptor. Equol possesses a strong affinity for estrogen receptors and induces transcription more strongly compared with other isoflavones (Morito *et al.* 2001). We speculated that the estrogen receptor may relate to the antibacterial activity displayed by equol, and thus, female hormones may also inhibit growth of *C. difficile*. However, we could not observe inhibitory activity by female hormones in our study. Therefore, equol's antimicrobial activity may not relate to its strong ability of binding estrogen receptor.

In Western countries only 25-30% of the adult population produces S-(-)equol when fed soy foods containing isoflavones. This is significantly lower than that the reported 50-60% frequency of equol-producers among adults from Japan, Korea, and China (Setchell *et al.* 2010; Oren *et al.* 2016). We speculate that the reasons for these differences in the frequency of equol-production between the different populations may be related to the type of soy foods consumed.

The incidence of 1.04 cases per 10,000 patient-days in japan was lower than that the rates of hospital-onset CDI reported in previous multicenter studies conducted in Western countries (4.1-8.4 cases per 10,000 patient-days) (Kobayashi *et al.* 2017). However, recent studies reported no considerable difference in the incidence of CDI (Kato *et al.* 2019); moreover, cases of CDI could be underdiagnosed. Mortality at 30 days has been reported to be 9–38% (Mitchell *et al.* 2012) in Western countries. On the other hand, in Japan, mortality at 30 days remains 6.9–15.1% (Honda *et al.* 2014; Takahashi *et al.* 2014; Mori *et al.* 2015), indicating some differences between Western countries and Japan in damage due to CDI. Several factors may contribute to the difference in incidence between Japan and Western countries. One reason is a difference in the prevalent type of *C. difficile* strain. Previous epidemiological studies in Japan showed a high prevalence of PCR ribotypes 018, 014, and 002. Meanwhile, sporadic nosocomial cases due to highly virulent strains, such as PCR ribotype 027, have been reported to cause CDI outbreak in the West (Kato *et al.* 2007; Sawabe *et al.* 2007). Although ribotype 027 was detected in Japan (Cornely *et al.* 2012; Leffler *et al.* 2015), CDI outbreak was not reported. Therefore, we consider that the equol produced from consumed soy foods could be associated with a decrease in damage due to CDI.

**Conflict of Interests**

Yumi Tanaka, first author of this manuscript, works for Otsuka pharmaceutical Co., Ltd. But there is no funding, item supplying and information from my company.

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| *Clostridium difficile* | tcdA | tcdB | cdtA | cdtB |
|---|---|---|---|---|
| BI/027/NAP1 | + | + | + | + |
| TUM12745 | - | + | - | - |
| TUM12806 | + | + | + | + |
| TUM12855 | + | + | + | + |

Table 1 Expression profile of toxin A, toxin B and binary toxin related gene in in *C. difficile*.

***Figure legends***

Figure 1. Dose-dependent response of equol on growth of *C. difficile* 027

Bacteria was cultured as described in Material and Methods and $OD_{600}$ of each culture was measured at indicated time points. Equol (dissolved in DMSO) was added at a final concentration of 100 μg $ml^{-1}$ (closed triangles) or 50 μg $ml^{-1}$ (closed circles) at time 0. The control (closed squares) contained only DMSO.

Figure 2. Effect of equol on growth of *C. difficile* various strains

Bacteria was cultured as described in Material and Methods and $OD_{600}$ of each culture was measured at indicated time points. Equol (dissolved in DMSO) was added at a final concentration of 100 μg $ml^{-1}$ (closed triangles) at time 0. The control culture (closed squares) contained only DMSO.

Figure 3. Effect of equol on toxinogenicity

Bacteria was cultured as described in Material and Methods and level of toxin in each culture was measured at indicated time points. Equol (dissolved in DMSO) was added at a final concentration of 100 μg $ml^{-1}$ (closed triangles) at time 0. The control culture (closed squares) contained only DMSO.

Figure 4. Effect of equol on spores forming and morphology of *C. difficile*

Bacterial was cultured as described Material and Methods and spores formed in each culture

were counted at indicated days (a). Equol (dissolved in DMSO) was added at a final concentration of 100 μg $ml^{-1}$ at time 0 (left bar). The control culture contained only DMSO (right bar). Precipitate of bacteria was separated and then stained as described in Methods. Image of cell culture containing equol is shown in (b) and that of control culture in (c). The long blue bars are vegetative cells of *C. difficile*. The small red rods are the spore of *C. difficile*.

Figure 5. Effect of equol on growth of *E. coli*, *C. perfrigens* and *B. fragilis*

Bacteria was cultured as described in Material and Methods and $OD_{600}$ of each culture was measured at indicated times. Equol (dissolved in DMSO) was added at a final concentration of 100 μg $ml^{-1}$ (closed triangles) at time 0. The control culture (closed squares) contained only DMSO.

Figure 6. Effect of isoflavones, daidzein and dihydrodaidzein, on growth of *C. difficile*

The metabolic pathway of daidzein carried out by intestinal flora and the structure of the metabolites are shown in (a). Bacteria were cultured as described in Methods and $OD_{600}$ of each culture was measured 24 h later (b).

Figure 7. Effect of female hormones on growth of *C. difficile*

Bacteria was cultured as described in Material and Methods and $OD_{600}$ of each culture was measured at indicated time points.

Figure 8. Chemical structures of isoflavones

The basic structure of isoflavone group is indicated. H-, OH- and $OCH_3$- residue are coordinated to the position R.