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Research article

Impact of prebiotics on equol production from soymilk isoflavones by two *Bifidobacterium* species

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ABSTRACT

The influence of commercial prebiotics (fructo-oligosaccharides and inulin) and sugars (glucose and sucrose) on enhancing equol production from soymilk isoflavones by *Bifidobacterium longum* BB536 and *Bifidobacterium breve* ATCC 15700 was evaluated *in vitro*. Sterilized soymilk was inoculated with each bacterial species at 37 °C for 48 h. The growth and β-glucosidase enzyme activity for the two *Bifidobacterium* species in soymilk throughout fermentation were assessed. The highest viable count for *B. breve* (8.75 log CFU/ml) was reached at 36 h and for *B. longum* (8.55 log CFU/ml) at 24 h. Both bacterial species displayed β-glucosidase activity. *B. breve* showed increased enzyme activity (4.126 U) at 36 h, while *B. longum* exhibited maximum activity (3.935 U) at 24 h of fermentation. Among the prebiotics screened for their effect in isoflavones transformation to equol, inulin delivered the highest effect on equol production. The co-culture of *B. longum* BB536 and *B. breve* ATCC15700 in soymilk supplemented with inulin produced the highest level (11.49 mmol/l) of equol at 48 h of fermentation process. Level of daidzin declined whereas that of daidzein increased, and then gradually decreased due to formation of equol when soymilk was fermented using bifidobacterial. This suggests that the nutritional value of soymilk may be increased by increasing bioavailability of the bioactive ingredients. Collectively these data identify probiotics and prebiotic combinations suitable for inclusion in soymilk to enhance equol production.

1. Introduction

A significant body of research has been directed to the nutritious and healthy properties of soybean and soy products. It has been found that soybean isoflavones and isoflavone-derived metabolites resemble estrogen and exhibit certain of its health benefits (Chen et al., 2018; Wee et al., 2017; Bilal et al., 2014). Isoflavones include aglycones and their glycosides (Hughes et al., 2003). It is important to clarify that aglycones (daidzein and genistein) are the more biologically active form of isoflavones than their glycosides (genistin, daidzin) (Elghali et al., 2012; Kawakami et al., 2005). Daidzein (7-hydroxy-3- (4-hydroxyphenyl)-4H-chromen-4-one) is one of the therapeutically important natural isoflavones originated in soybean. Daidzein has been approved for relieving menopausal syndromes in females, treatments of hypertension, coronary heart disease, cerebral clotting, dizziness, and deafness. However, daidzein does not commonly show the estrogenic activity unless it is converted to equol by the intestinal bacteria (Wang et al., 2017). Equol (4′, 7-isoflavandiol) is an isoflavone metabolite derived from daidzin/daidzein by certain bacterial biotypes in small intestine and colon of human, has non-planar construction which offers its physiological properties (Raffi, 2015; Del Rio et al., 2013; Setchell and Clerici 2010). It is more stable, more easily absorbed, and has stronger estrogenic activity than the other isoflavones or its precursor molecule daidzein (Jackson et al., 2011; Setchell et al., 2005).

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In addition, equol has been confirmed as having a protective action on osteoporosis by up regulating the minerals content and bones density in menopausal women (Lambert et al., 2017) (*S*)-Equol exhibits potential neuro-protective effects when it was used by Alzheimer's patients (Wilkins et al., 2017). About 25–30% of younger individuals are able to produce equol *in vivo* when fed with soy bean products. Thus, there is a need to improve the methods used for equol production. One of promising equol production approaches is natural bacterial fermentation. However, lower growth and productivity are the major problems of this procedure which should be resolved (Li, 2019).

Bifidobacterium species are reported to exhibit health-promoting effects and are classified as probiotic organisms since they are thought to enhance the bacterial homeostasis in the human digestive tract (Schrezenmeir and de Vrese, 2001). Probiotics possess several healthy features, including antimicrobial and anticarcinogenic activities as well as other valuable health effects to the host (Lourens-Hattingh and Viljoen, 2001). Soymilk helps on delivering probiotic to the consumer (Otieno et al., 2005). Moreover, studies reported that, soymilk is a good culture medium for bifidobacterial growth. This is for the reason that it consists of various carbohydrates, sucrose, raffinose, glucose and stachyose which are fermented by the majority of strains affiliated to this genus (Liu, 1997; Desjardins et al., 1990). However, humans are not able to produce sufficient amounts of α-galactosidase (an enzyme that catalyzes breakdown of the terminal α-galactosyl moieties of polysaccharides and oligosaccharides, in the digestive system to completely digest the galactosaccharides of soymilk. Therefore, bacterial metabolism of these α-galactosyl oligosaccharides requires strains with higher α-galactosidase activity (Lu-Kwang et al., 2018; Sengupta et al., 2015).

A prebiotic is identified as "a substrate that is selectively utilized by host microorganisms conferring a health benefit. This definition expands the idea of prebiotics to possibly include non-carbohydrate substances, applications to body sites other than the gastrointestinal tract, and diverse categories other than food (Gibson et al., 2017). Since the major influence of prebiotics is to stimulate bacterial growth and/or activity, primarily *Bifidobacteria* have a role in promoting human health condition (Park et al., 2016; Kaur and Gupta 2002; Gibson and Roberfroid, 1995). Besides, prebiotics (FOS and inulin) are recognized to have influence on development of *Lactobacillus* and/or *Bifidobacterium* spp. Therefore, supplementation of soymilk with prebiotic could enhance bacterial growth in soymilk by offering additional supply of oligosaccharides. Furthermore, fructo-oligosaccharides (FOS), inulin and galacto-oligosaccharides (GOS) have attracted wide attention because they are appropriate food for *Bifidobacteria* in the intestine and can enhance the stability of useful bacteria in the gut, therefore they can improve human's health (Simpson and Campbell, 2015; Huebner et al., 2007; Tuohy et al., 2003). A study by Roberfroid et al. (1998) stated that the inulin-type fructans are the only prebiotics characterized as functional food ingredients; however another one reported that prebiotics with specific standard (in *in vivo* and *in vitro* experiments) effective features include inulin, fructo-oligosaccharides (FOS) and galacto-oligo-saccharides (GOS) (Florowska et al., 2016).

In the present study, soymilk was used as a natural source of isoflavones, so it is better to explain that, selection of bacterial species for screening of equol production from soymilk was created depending on β-glucosidase activity of bacterial species. Due to our interest in β-glucosidase enzyme, this study only included screening of the β-glucosidase activity as it is essential for enzymatic transformation of isoflavone glycosides to aglycones to provide excessive levels of daidzein, the direct precursor of equol (Yuksekdag et al.,2017; Otienoet al., 2006; Tsangalis et al., 2002). Also this study evaluated (*in-vitro*) the influence of two commercial prebiotics (fructo-oligosaccharides and inulin) and two sugars (glucose and sucrose) on equol production from soymilk isoflavones by *Bifidobacterium longum* BB536 and *Bifidobacterium breve* ATCC15700.

2. Materials and methods

2.1. Materials

All standards (daidzein, equol and daidzin) were bought from Millipore Sigma Chemical Co (St. Louis, USA). Soybean (*Glycine max* (L.) Merrill) was bought from the local market in Serdang-Selangor, Malaysia. The chemicals of analytical HPLC grade were purchased from Merck (Darmstadt, Germany). Brain Heart Infusion (BHI) broth was used for motivation of bacterial strains. It was handled in compliance with the manufacturing instructions (Oxoid Ltd., West Heidelberg/Vic., Australia). Glucose as well as Sucrose was from Millipore Sigma (Louis, USA), while Inulin and Fructo-oligosaccharides from Orafti Pty.Ltd,(-Tienen, Belgium).

2.2. Methods

2.2.1. Bifidobacteria culture conditions

Unadulterated cultures of *B. breve* ATCC 15700and *B. longum* BB536 were used. Gram staining was used to check the purity of bacterial cultures. The standard bacterial culture was proliferated and stored in 40% glycerol at −80 °C for further use. Bifidobacteria grow anaerobically. Anaerobic environment was obtained with Anaero Gen sachets (Oxoid Ltd., West Heidelberg/Vic., Australia).

2.2.2. Production of soymilk

Soymilk was produced following the procedure described by Hou et al. (2000) with few changes. Soybean grains were firstly cleaned up and soaked overnight in distilled water. The soaked soybeans were added to ten times the weight of (100 g dry soya bean to 1000 ml water) distilled water and boiled for 30 min at 95 °C in a water bath. Further it was blended for 5 min. The obtained slurry was then purified through double-layered cheesecloth to yield soymilk (New England Cheese making supply company, South Deerfield, MA, USA). Soymilk was autoclaved at 121 °C for 15 min and stored in a refrigerator (4 °C).

2.2.3. Enumeration of bacterial population

Viable cell counts of *B. breve* and *B. longum* were established in duplicate using the pour plate method on BHI agar medium. Each fermented soymilk was added to 90 ml sterile 0.85% saline (w/v) and vortexed for 30 s. Resultant suspension was serially diluted with sterile 9ml saline and 1 ml of the proper dilution was used for selective enumeration by the pour plate technique. The cell growth of each organism was assessed by enumerating a bacterial population on BHI agar at 0, 12, 24, 36 and 48 h of fermentation. To be effective, plates containing 30–300 colonies were counted and recorded as CFU per ml of fermented soymilk.

2.2.4. Preparation of bacterial single and co-culture inoculums

Bacterial species (*B. breve* ATCC 15700, *B. longum* BB536) were activated in BHI medium by relocating three times in 10 ml of BHI broth and incubation at 37 °C 20 h followed by collecting bacterial cells by centrifuging (3000 × *g* for 15 min). To get bacterial co-culture cell suspensions, the two cell suspensions were mixed at a volume ratio of 1:1. Inoculums of the bacterial single and co-culture were set by using 100 ml of sterile soymilk and incubation for 20 h at 37 °C.

2.2.5. β-glucosidase activity assay

B. longum BB536 and *B. breve* ATCC15700 were activated by incubating in 10 ml of BHI broth. Incubation was carried out at 37 °C for 20 h. Bacterial cells were collected by centrifugation at 3000 × g for 15 min. The inoculum of single culture for every bacteriological strain was made with 50 ml of sterile soymilk and incubation for 20 h at 37 °C. Ten milliliters of the vigorous culture were injected into 250 ml of each

Table 1. Growth of *B. breve* ATCC15700 and *B. longum* BB536 during fermentation of soymilk for 48 h at 37 °C.

Time/h	log CFU/ml	
	B. breve	*B. longum*
0	4.60 ± 0.03^{a}	4.61 ± 0.01^{a}
12	6.58 ± 0.02^{b}	6.45 ± 0.04^{b}
24	7.50 ± 0.01^{c}	8.55 ± 0.02^{c}
36	8.75 ± 0.02^{d}	7.35 ± 0.01^{d}
48	6.28 ± 0.01^{b}	6.18 ± 0.02^{b}

Values are means of log CFU/ml in soymilk during the fermentation time ±standard deviation. Means in the same column with different superscripts letters are significantly different ($P \leq 0.05$).

soymilk (5% v/v) batches of and incubated for 48 h at 37 °C. Fifty milliliters were withdrawn aseptically from every inoculum at 12, 24, 36 and 48 h of incubation to measure the enzyme activity. β-Glucosidase activity of the bacterial strains was evaluated by identifying the degree of hydrolysis of the substrate ρ-NPG. It was prepared in 100 mM sodium phosphate buffer (pH 7.0) (Millipore Sigma, Chemical Co., St. Louis, Mo–U.S.A). One milliliter of ρ NPG (5 mM) was added to 10 ml of each aliquot and incubated at 37 °C for 30 min (Otieno et al., 2006; Scalabrini et al., 1998). The reaction was ended by adding of 500 μl from 1 M cold sodium carbonate. The aliquot was transferred to centrifuge tube followed by centrifugation (14,000 g for 30 min) using Eppendorf refrigerated centrifuge (Model 5810 R). The quantity of ρ-nitro-phenol relieved was determined by Perkin Elmer spectrophotometer (Model: Lambda 25 UV/VIS Spectrophotometer) at 420 nm. One unit of the enzyme was defined as the amount of enzyme that released1 μ mol of ρ-nitro-phenol from the substrate ρ NPG, per ml per min under assay conditions.

2.2.6. Batch fermentation conditions

The fermentation process was executed in 1 L volume bioreactor BIOSTAT QDCU3 (Sartorius BBI System GmbH, Melsungen, Germany) and controlling of temperature was achieved using water bath (Jeio Tech Desk Top, Seoul, South Korea) and an electronic stirrer (Gas-Col Ltd, Northvale, NJ 07647, USA). The temperature was set at 37 °C. Anaerobic condition for fermentation was conserved by flushing oxygen-free nitrogen gas through the medium. No control stood for pH. The stirring speed for all batch fermentation was set at 200 rpm/min. One hundred ml inoculums of single culture for each bacterial strain (*B. longum* BB536 and *B. breve* ATCC 15700) in sterile soymilk were transferred to the fermenter to inoculate the soymilk in a 2-L vessel (with 1 L working volume). Samples of fermented soymilk were taken at 0, 24 and 48 h into sterile universal bottles to examine changes on isoflavones concentrations.

2.2.7. Sample preparation for isoflavones investigation by high performance liquid chromatography (HPLC)

Fermented soymilk (2 ml) was added to 80% methanol (8 ml) and stirred for 2 h at 25 °C.

Then, the blend was centrifuged at 9000 rpm for 20 min. The supernatant was clarified using a 0.22 μm syringe membrane into HPLC vials and kept at -20 °C for HPLC investigation.

2.2.8. High performance liquid chromatography (HPLC) protocol

HPLC protocol was in accordance with the method mentioned by Elghali et al. (2012) with some alterations. Twenty microliters of sample were injected into high-performance liquid chromatography (HPLC) (Model CO-2065 JASCO Corporation Hachioji, Tokyo, Japan) equipped with C18 reversed-phase column (25 cm × 4.5 cm × 5 μ) (Ascentis–Supelco, Sigma-Aldrich Co. LLC. L, USA), diode array ultraviolet (UV) visible detector, vacuum degasser, and thermostatically controlled column compartment. Column temperature was set at 27 °C. HPLC gradient elution was composed of 10% acetonitrile solution in water (solution A) and 90% acetonitrile solution in water (solution B). The elution program was as follows: solution B was run at 30% for 15 min, linearly increased to 50% for 10 min, and then linearly increased to 70% for 5 min. The flow rate was at 1 ml/min. A diode array UV-visible detector was set at 270 nm. UV spectra and retention times of the metabolites produced from daidzin and daidzein by bacteria were compared with those of the standard compounds daidzin, daidzein and equol in HPLC chromatograms.

2.2.9. Screening of prebiotics for equol production

Commercial sugars and prebiotics were screened for ability to enhance equol production from fermented soymilk. They were: glucose (≥99.5%) and sucrose (≥99.5%) purity [Sigma, Louis, USA], inulin and fructo-oligosaccharides (OraftiPty. Ltd, Tienen, Belgium). The inulin used was Raftiline ST with a purity of 92% and an average degree of polymerization (DP) of 10. The fructo-oligosaccharide (FOS) which utilized was Raftilose P95 that formed from 5% of glucose, fructose and sucrose. It also composed of oligo-fructose with DP ranging from 2-7 with an average of 4. One hundred ml of sterile soymilk supplemented with Inulin, FOS, Glucose and Sucrose (1%w/v) individually was inoculated with activated culture of (*B. breve* ATCC15700 and *B. longum* BB536) and incubated anaerobically at 37 °C for 48 h. The soymilk medium was set to contain a final concentration 1% (w/v). Trials of inoculated soymilk were taken at 12, 24, 36 and 48 h to measure the quantity of isoflavones by the usage of HPLC (see section 2.2.8).

3. Statistical analysis

Results analysis was performed using SPSS version 16. Data achieved were subjected to analysis of variance (ANOVA) and minimum significant difference tests (LSD). Fisher test was used to classify the significant differences among mean values ($P \leq 0.05$).

4. Results and discussion

4.1. Cell growth during fermentation

Growth of *B. breve* and *B. longum* in soymilk during fermentation was assayed by enumerating the viable cell counts. Table 1 shows the growth pattern of *B. breve* and *B. longum* at 0, 12, 24 and 48 h in soymilk during fermentation at 37 °C. The highest viable counts for *B. breve* (8.75log CFU/ml)and *B. longum* (8.55 log CFU/ml) was reached at 36 and 24 h, respectively. These findings agreed with those showed that different lactic acid bacteria strains revealed greater (7–9 log CFU/ml) cell population in soymilk (Rekha, &Vijayalakshmi, 2011; Chun et al., 2007). Moreover, after 48 h there was dropping on *B. breve* and *B. longum* growth, which clarified the conversion from exponential to stationary growth phase. The diminution in population was 2.47 and 2.37 log CFU/ml, respectively, over 48 h of incubation. Reduction in the growth of bifidobacteria at 48 h fermentation is probably owing to shortage of nutrient supply in the medium, which is strongly supported by Rekha, &Vijayalakshmi (2011) and Scalabarini et al. (1998), who found that the nutrient content of soymilk is reduced at 48 h fermentation with Bifidobacteria, fully to one-half of the original concentration. Donkor and Shah (2008) stated that the maximum viable count took place at 12 h for *L. casei* L26, 24 h for *B. lactis* B94, and 36 h for *L. aciophilus* L10. However, the cell growth in soymilk fermentation is influenced by the cultures and fermentation period (Jiyeon et al., 2008).

4.2. β-Glucosidase activity of Bifidobacterium species in fermented soymilk

β-Glucosidase activity of soymilk fermented with *Bifidobacterium* species is shown in Table 2. Both bacterial species exhibited measurable levels of the enzyme activity. The enzyme activity differed between the

Table 2. β-Glucosidase activity of *B. breve* ATCC 15700 and *B. longum* BB536 in soymilk fermented for 48 h at 37 °C.

Incubation time (h)	*B. breve*	*B. longum*
12	2.208 ± 0.20^{a}	2.683 ± 0.24^{a}
24	3.365 ± 0.19^{b}	3.935 ± 0.79^{b}
36	4.126 ± 0.33^{c}	3.186 ± 0.05^{b}
48	2.314 ± 0.39^{a}	2.015 ± 0.03^{c}

Values were means ± standard deviation (SD) of units of enzymes (n = 7).a–c Means in the same column with different superscripts are significantly different ($P \leq 0.05$). One unit of enzyme (U) is the amount of β-glucosidase that released one μ molar of ρ-nitrophenol from ρ-NPG per ml/min at 37 °C.

tested organisms. Moreover, there was a significant difference ($P \leq 0.005$) in β-glucosidase activity at the duration of 48 h for the fermented soymilk. However, the maximum enzyme activity for *B. breve* (4.126 U) and *B. longum* (3.935 U) was achieved at 36 and 24 h of fermentation, respectively. This is similar to the findings reported by Rekha, &Vijayalakshmi (2011) and Otieno et al. (2005) who mentioned that probiotic bacteria (*Bifidobacterium* and *Lactobacillus*) are known to display strain-dependent β-glucosidase activity in soymilk. However, relied upon β-glucosidase activity in soymilk, it seemed that *L. acidophilus* and *L. casei* strains presented superior β-glucosidase activity (2.204; 2.199 U), respectively, to that of *B. animalis* BB12 (2.095 U), *B. longum* 20099 (1.998U) and *B. longum 536* (1.972U) (Otieno et al., 2005). Mostly; β-glucosidase activity was established to be reliant on time and strain. It is notices that, soymilk fermented with *B. breve*, which had the maximum β-glucosidase activity (4.126 U) at 36 h of fermentation, represented the highest cell number (8.75 log CFU/ml) also at 36 h. Similarly, soymilk fermented with *B. longum* which has the highest β-glucosidase activity (3.935 U) at 24 h of fermentation, had a maximum cell number (8.55 log CFU/ml) at 24 h of fermentation. Therefore, increased cell growth may be followed by an increase in enzyme activity. It appears that there is a correlation between β-glucosidase activity and growth characteristics during fermentation of soymilk. So, the decrease in β-glucosidase activity at 48 h might be due to decline of the bacterial growth at 48 h of fermentation time (Table 1). These findings agreed with those of Donkor and Shah (2008) who stated that there is a parallel relationship between growth of microorganisms in soymilk and β-glucosidase activity. Otieno et al. (2005) stated that, the increase in β-glucosidase activity and the subsequent decline apparently corresponded to the growth of these probiotic microorganisms in the soy media (growth results not shown). However, the tested bacterial strains revealed an increase in β-glucosidase activity upon incubation time of up to 24 h followed by reduction as fermentation progressed. Three strains of *L. acidophilus* and two strains of *L. casei* exhibited increasing β-glucosidase activity up to 24 h and declining as fermentation proceeded. According to the result achieved from this research which was intended for the screening of β-glucosidase enzyme activity of different bacterial species, *B. breve* ATCC 15700 and *B. longum* BB536 exhibited different β-glucosidase activity through incubation in soymilk for 48 h. Accordingly, β-glucosidase activity is strain reliant and differs amongst the organisms. In addition, Donkor and Shah (2008)reported that, *L. acidophilus* L10, displayed higher β-glucosidase activity, when comparing to *B. lactis* B94 and *L.casei* L26. Moreover, another study found that *Lactobacillus acidophilus* exhibited the highest β-glucosidase activity at 24 h of fermentation in soymilk compared to *Bifidobacterium* spp. and *L. casei* (Otieno et al., 2006).Furthermore, Bifidobacteria species showed different levels of β-glucosidase yields dependent on the sugar quantity for the cultivation media required by the species and to the phase of growth (Tsangalis et al., 2002).

4.3. Concentrations of isoflavones in plain soymilk fermented with two bacterial species

As presented in Table 3, the amounts of isoflavones isomers are not significantly changed and equol was not found in plain soymilk.

Moreover, the level of isoflavones glucosides (daidzin) was significantly declined when soymilk fermented with *B. breve*. The levels of daidzin at 0, 24 and 48 h were 10.36 ± 0.02, 8.45 ± 0.03 and 7.38 ± 0.01 mmol/l, respectively. Instead, the concentrations of daidzein increased significantly through fermentation of soymilk with *B. breve*. However, at 0 h, the concentration of daidzein was 1.48 ± 0.02 and after 12 h of incubation it was 6.61 ± 0.02 mmol/l, then it was followed by gradually decrease in the concentrations due to production of equol. Moreover, at 0 h, equol was not detected, after 12 h it was 0.56 ± 0.04 and then increased regularly to2.23 ± 0.04 mmol/l after 48 h of incubation time. Furthermore, once soymilk was fermented with *B. longum*, the concentrations of daidzin were decreased significantly from 10.35 mmol/l at 0 h to 7.15 mmol/l after 48 h of incubation period. In contrast daidzein concentrations were increased from 1.47 at 0 h to 7.34 mmol/l after 24 h. Later, it started to decrease slowly after 36 h owing to equol production.

Table 3. Concentrations of isoflavones isomers (mmol l^{-1}) in plain soymilk fermented by two bacterial species at 0, 12, 24, 36 and 48 h of incubation at 37 °C.

Treatment	Time(h)	Isoflavones isomers		
		Daidzin	Daidzein	Equol
Soymilk-	0	10.36 ± 0.11^{a}	1.48 ± 0.03^{a}	ND
(control)	12	10.38 ± 0.05^{a}	1.46 ± 0.06^{a}	ND
	24	10.35 ± 0.03^{a}	1.48 ± 0.04^{a}	ND
	36	10.37 ± 0.03^{a}	1.49 ± 0.10^{a}	ND
	48	10.38 ± 0.02^{a}	1.47 ± 0.04^{a}	ND
B. breve	0	10.36 ± 0.02^{a}	$1.\ 48 \pm 0.02^{a}$	ND
	12	9.34 ± 0.02^{b}	$6.\ 61 \pm 0.02^{b}$	0.56 ± 0.01^{a}
	24	8.45 ± 0.03^{c}	5.94 ± 0.02^{b}	1.38 ± 0.01^{b}
	36	7.58 ± 0.03^{d}	4.11 ± 0.03^{c}	1.74 ± 0.02^{b}
	48	7.38 ± 0.01^{d}	3.02 ± 0.01^{d}	2.23 ± 0.04^{c}
B. longum	0	10.35 ± 0.0^{a}	1.47 ± 0.04^{a}	ND
	12	9.45 ± 0.05^{b}	4.63 ± 0.05^{b}	0.22 ± 0.01^{a}
	24	8.46 ± 0.05^{b}	7.34 ± 0.01^{c}	0.89 ± 0.05^{b}
	36	7.48 ± 0.07^{c}	6.23 ± 0.04^{d}	1.88 ± 0.06^{c}
	48	7.15 ± 0.01^{c}	5.18 ± 0.02^{f}	2.01 ± 0.03^{c}

Values are Means of concentrations of isoflavones in soymilk during the fermentation period± standard deviation. Means in the same column with different superscripts letters are significantly different ($P \leq 0.05$). Values <0.01 are considered to be not detected (ND).

Table 4. Concentration of isoflavones (mmol l^{-1}) in soymilk supplemented with Sucrose and Glucose and fermented with single and co-culture of *B. breve* and *B. longum* BB536for 48 h 37 °C.

Bacteria species	Time/h	Sugars					
		Sucrose			Glucose		
		Daidzin	Daidzein	Equol	Daidzin	Daidzein	Equol
B. breve	12	10.22 ± 0.02^{a}	7.54 ± 0.02^{a}	2.40 ± 0.01^{a}	10.54 ± 0.02^{a}	6.40 ± 0.01^{a}	3.22 ± 0.02^{a}
	24	8.34 ± 0.02^{b}	8.61 ± 0.02^{b}	2.84 ± 0.01^{a}	9.61 ± 0.02^{b}	6.84 ± 0.01^{b}	3.34 ± 0.02^{a}
	36	6.45 ± 0.03^{c}	6.37 ± 0.02^{c}	3.59 ± 0.01^{b}	8.97 ± 0.02^{c}	5.59 ± 0.01^{c}	3.45 ± 0.03^{b}
	48	5.58 ± 0.03^{d}	5.94 ± 0.03^{d}	4.11 ± 0.02^{c}	8.14 ± 0.03^{d}	3.81 ± 0.02^{d}	3.58 ± 0.03^{b}
B. longum	12	10.17 ± 0.01^{a}	6.14 ± 0.04^{a}	2.74 ± 0.02^{a}	10.44 ± 0.04^{a}	6.74 ± 0.02^{a}	2.17 ± 0.01^{a}
	24	9.45 ± 0.05^{b}	7.63 ± 0.05^{b}	2.95 ± 0.11^{a}	9.83 ± 0.05^{b}	5.95 ± 0.11^{b}	3.45 ± 0.05^{b}
	36	7.46 ± 0.05^{b}	6.37 ± 0.06^{c}	3.39 ± 0.05^{b}	9.37 ± 0.06^{b}	3.39 ± 0.05^{c}	3.46 ± 0.05^{b}
	48	5.48 ± 0.07^{b}	5.98 ± 0.04^{d}	3.88 ± 0.06^{b}	8.68 ± 0.04^{c}	2.88 ± 0.06^{d}	3.48 ± 0.07^{b}
B. breve + *B. longum*	12	7.26 ± 0.025^{a}	8.44 ± 0.053^{a}	3.32 ± 0.01^{a}	10.14 ± 0.05^{a}	7.72 ± 0.02^{a}	4.2 ± 0.025^{a}
	24	6.32 ± 0.067^{b}	9.50 ± 0.023^{b}	5.97 ± 0.03^{b}	8.50 ± 0.02^{b}	6.97 ± 0.03^{b}	5.32 ± 0.07^{b}
	36	5.10 ± 0.017^{c}	6.78 ± 0.043^{c}	6.61 ± 0.03^{c}	7.78 ± 0.04^{c}	5.61 ± 0.03^{c}	5.90 ± 0.02^{c}
	48	4.33 ± 0.035^{d}	3.63 ± 0.029^{d}	7.31 ± 0.06^{d}	6.63 ± 0.03^{d}	4.31 ± 0.06^{d}	6.33 ± 0.04^{d}

Values are Means of concentrations of isoflavones in soymilk during the fermentationperiod ±standard deviation. Means in the same column with different superscripts letters are significantly different ($P \leq 0.05$) .

4.4. Effect of prebiotics on equol production

In the current research the effects of the selected prebiotics such as (inulin, FOS) and glucose and sucrose on equol production from soymilk isoflavones using different bacterial species *(B.longum*BB536 and *B. breve* ATCC 15700) were estimated. Table (3) shows the results of plain soymilk fermentation with *B. longum* BB536 and *B. breve* ATCC 15700. There was noticeable decrease in isoflavone glycoside (daidzin) and daidzein parallel to increasing of equol production by fermentation time.

Table 4 represents the influence of adding sucrose to soymilk on equol production. As shown, by 48 h of incubation, *B. longum* BB536 and *B. breve* ATCC 15700 co-culture delivered high quantity of equol (7.31 mmol/l); this amount is high compared to that being produced in the case of plain soymilk. These findings go along with those demonstrated by Wei et al. (2007), which revealed that supplementation of soymilk with sucrose for isoflavones aglycones and equol production using five strains of isoflavones metabolizing microorganism, yielded smaller quantities of aglycones and equol than those observed when soymilk was enriched with fructose and lactose sugars. Results for the effect of glucose addition on soymilk fermented with single and co-culture of *B. breve* ATCC 15700 and *B. longum* BB536 for 48 h were also displayed in Table 4. The results showing that, there is no significant different in the amounts of daidzin, daidzein and equol in soymilk supplemented by glucose compare to those of the plain soymilk during the fermentation time. This finding is consistent with that of Tsangalis et al. (2002) who stated that, the concentrations of daidzin; daidzein and equol after 48 h incubation of 4 strains of *Bifidobacterium* in soymilk supplemented with glucose were approximately the same in complemented soymilk and in ordinary soymilk by 24 h of fermentation. The effect of supplementation of soymilk by FOS on equol production is varying within the Bifidobacteria species (Table 5). *B. breve* ATCC 15700 showed high amount (4.94 mmol/l) of equol after 48 h incubation period comparing to plain soymilk. Co-culture from *B. breve* ATCC 15700 and *B. longum* BB536 showed high level (8.63 mmol/l) of equol after 48 h incubation period. These findings remained parallel to those published by Uehara et al. (2001), who disclosed that the growth of bifidobacteria and furthermore the transformation of isoflavone conjugate to produce the correspondence aglycones and equol can be stimulated by FOS. The present results also agree with the finding that addition of FOS to soymilk professionally and significantly ($P \leq 0.05$) increases the β-glucosidase activity, and this was

Table 5. Concentrations of isoflavones (mmol l^{-1}) in soymilk supplemented with FOS inulin and fermented with single and co-culture of *B. breve* and *B. longum* BB536 for 48 h 37 °C.

Bacteria species	Time (h)	Prebiotics					
		FOS			Inulin		
		Daidzin	Daidzein	Equol	Daidzin	Daidzein	Equol
B. breve	12	9.43 ± 0.01^{a}	7.77 ± 0.01^{a}	3.54 ± 0.02^{a}	7.62 ± 0.02^{a}	9.40 ± 0.01^{a}	3.76 ± 0.06^{a}
	24	7.36 ± 0.01^{b}	9.85 ± 0.02^{b}	3.61 ± 0.02^{b}	6.34 ± 0.02^{b}	10.84 ± 0.01^{b}	4.24 ± 0.06^{b}
	36	6.91 ± 0.01^{c}	10.84 ± 0.02^{c}	4.37 ± 0.02^{c}	4.45 ± 0.03^{c}	7.59 ± 0.01^{c}	5.06 ± 0.06^{c}
	48	5.97 ± 0.01^{d}	8.17 ± 0.03^{d}	4.94 ± 0.03^{d}	2.58 ± 0.03^{d}	4.11 ± 0.02^{d}	6.79 ± 0.01^{d}
B. longum	12	9.39 ± 0.01^{a}	8.14 ± 0.02^{a}	2.14 ± 0.04^{a}	9.17 ± 0.01^{a}	7.74 ± 0.02^{a}	2.12 ± 0.03^{a}
	24	7.56 ± 0.05^{b}	8.95 ± 0.11^{b}	2.63 ± 0.05^{b}	8.45 ± 0.05^{b}	8.95 ± 0.11^{b}	3.46 ± 0.02^{b}
	36	6.03 ± 0.05^{b}	9.39 ± 0.05^{c}	3.37 ± 0.06^{c}	6.46 ± 0.05^{b}	6.39 ± 0.05^{c}	3.91 ± 0.06^{c}
	48	5.48 ± 0.07^{b}	7.88 ± 0.06^{d}	3.98 ± 0.04^{d}	4.48 ± 0.07^{b}	5.88 ± 0.06^{d}	4.38 ± 0.10^{d}
B. breve + *B. longum*	12	8.2 ± 0.025^{a}	10.32 ± 0.02^{a}	4.44 ± 0.05^{a}	6.2 ± 0.025^{a}	10.32 ± 0.02^{a}	6.82 ± 0.02^{a}
	24	6.32 ± 0.07^{b}	9.97 ± 0.03^{b}	5.50 ± 0.02^{b}	5.32 ± 0.07^{b}	9.97 ± 0.03^{b}	8.61 ± 0.04^{b}
	36	5.90 ± 0.02^{c}	6.61 ± 0.03^{c}	6.78 ± 0.04^{c}	3.90 ± 0.02^{c}	6.61 ± 0.03^{c}	9.27 ± 0.04^{c}
	48	4.33 ± 0.04^{d}	3.31 ± 0.06^{d}	8.63 ± 0.03^{d}	1.53 ± 0.04^{d}	2.31 ± 0.05^{d}	11.49 ± 0.36^{d}

Values are Means of concentrations of isoflavones in soymilk during the fermentation period ±standard deviation. Means in the same column for particular species with different superscripts letters are significantly different ($P \leq 0.05$).

Table 6. Concentration of equol (mmol l^{-1}) in soymilk supplemented with different carbohydrates and fermented with single and co-culture of *B. breve* ATCC 15700 and *B. longum* BB536 for 48 h.

Bacteria species	Time/h	SM + Glucose	SM + Sucrose	SM + FOS	SM + Inulin
B. breve	12	2.52 ± 0.02^{a}	1.40 ± 0.01^{a}	3.54 ± 0.02^{a}	3.76 ± 0.06^{a}
	24	3.34 ± 0.02^{b}	2.84 ± 0.01^{b}	3.61 ± 0.02^{b}	4.24 ± 0.06^{b}
	36	3.45 ± 0.03^{b}	3.59 ± 0.01^{c}	4.37 ± 0.02^{c}	5.06 ± 0.06^{c}
	48	3.58 ± 0.03^{b}	4.11 ± 0.02^{d}	4.94 ± 0.03^{d}	6.79 ± 0.01^{d}
B. longum	12	2.17 ± 0.01^{a}	2.74 ± 0.02^{a}	2.14 ± 0.04^{a}	2.12 ± 0.03^{a}
	24	3.45 ± 0.05^{b}	2.95 ± 0.11^{b}	2.63 ± 0.05^{b}	3.46 ± 0.02^{b}
	36	3.46 ± 0.05^{b}	3.39 ± 0.05^{c}	3.37 ± 0.06^{c}	3.91 ± 0.06^{c}
	48	3.48 ± 0.07^{b}	3.88 ± 0.06^{d}	3.98 ± 0.04^{d}	4.38 ± 0.10^{d}
B. breve + *B. longum*	12	4.22 ± 0.03^{a}	4.32 ± 0.02^{a}	4.44 ± 0.05^{a}	6.82 ± 0.02^{a}
	24	5.32 ± 0.07^{b}	5.97 ± 0.03^{b}	5.50 ± 0.02^{b}	8.61 ± 0.04^{b}
	36	5.90 ± 0.02^{c}	6.61 ± 0.03^{c}	6.78 ± 0.04^{c}	9.27 ± 0.04^{c}
	48	6.33 ± 0.04^{d}	7.31 ± 0.06^{d}	8.63 ± 0.03^{d}	11.49 ± 0.36^{d}

Values are Means of concentration of equol during the 48 h fermentation period ±standard deviation. Means in the same column for particular species with different superscripts letters are significantly different ($P \leq 0.05$). SM = Soymilk.

dominant in soymilk fermented with *L. acidophilus* (Yeo and Liong 2010) and with Ohta et al. (2002) who reported FOS enhanced cecal β-glucosidase action and daidzein conversion to equol in both OVX and SH mice. Consequently, these finding viewed that, FOS increased the growth of bacteria species responsible for the transformation, β-glucosidase activity and subsequently the bioavailability of isoflavones. Alternatively, Decroos et al. (2005) and Zafar et al. (2004) established that addition of fructo-oligosaccharides to the food could be a reason for equol production inhibition. As the digestion of FOS by gastrointestinal bacteria result in a great relief of hydrogen, the incidence of FOS possibly will change the colonic Microbiota and destroy the bacteria accountable for equol production and at the same time initiates alteration in hydrogen utilization; therefore, daidzein may not be metabolized to dihydrodaidzein or equol. The present results indicate that, addition of FOS and sucrose to soymilk significantly ($P \leq 0.05$) increases equol production from daidzein in fermented soymilk. Instead, Tsuji et al. (2010) confirmed that the addition of FOS or sucrose to soymilk significantly inhibited equol production by the human isolated bacterium *Slackia* sp. Strain NATTS. The results demonstrating the influence of inulin in transformation of isoflavones to produce equol are shown in table (5). It was noticed addition of inulin to soymilk offered the highest (co-culture = 11.49 mmol/l) amount of equol among both single and co-culture comparing to other carbohydrates added to soymilk. However, these findings are differing from those established by Zafar et al. (2004), who published that the absorption and concentrations of plasma equol were affected negatively by inulin. Levels of equol in serum were significantly lesser in the group nourished in inulin relative to that nourished in inulin free isoflavones diets. Another study revealed that inulin exhibited the greatest impact in hydrolyzing the malonyl daidzin, and this was most dominant in soymilk fermented by *Bifidobacterium* FTDC 8943 ($P < 0.05$). Addition of inulin to soymilk is significantly ($P < 0.05$) reduced the level of malonyl daidzin in soymilk fermented with *Bifidobacterium* FTDC 8943 about 49.3 % (Yeo and Liong 2010). Moreover, a study was described that ingestion of soy isoflavones with inulin for 21 days result on increases of plasma daidzein concentration in postmenopausal women compared with intake of intake of soy isoflavones without inulin (Zafar et al., 2004). This indicated that inulin has an influence on transformation of isoflavones glucosides via enhancing the growth of the colonic bacteria and therefore increasing the amount and activity of the bacterial enzymes responsible for isoflavones metabolism in the gut and besides increases their absorption and bioavailability (Piazza et al., 2007). Yet, these results agreed with our finding which showed that the high rate of conversion of daidzin to daidzein when inulin was added to soymilk medium during the fermentation process which made daidzein (the primary precursor of equol) more available. Table 6 summarizes the results for equol produced in fermented soymilk. The amount of equol produced by single culture (*B. breve* ATCC 15700/*B. longum* BB536) was less than that produced when fermentation was carried out with the co-culture of *B. breve* ATCC 15700 and *B. longum* BB536.The co-culture promotes high rates of β-glucosidase hydrolysis to aglycones than a single bacterial culture. Also it may offer nutrients and circumstances that someway preserve the sustainability of the other bacteria in the mixture of cultures (Garro et al., 2004).

5. Conclusion

Estimation of β-glucosidase activity for bacterial species found that, both bacterial species tested can generate different levels of β-glucosidase activity according to fermentation time. However, *B. breve*ATCC15700 exhibited maximal β-glucosidase activity at 36 h, while *B. longum* BB536 got it by 24 h of fermentation period (48 h) in soymilk. Therefore, the hydrolytic ability and enzyme activity could be unique for each strain. These results enhance our understanding of the impact of prebiotics on equol production from soymilk isoflavones. However, the results established that, all tested prebiotics had significant effect in equol production, but inulin exhibited the highest level of equol production comparing to FOS. So it was recommended that, in order to gain high levels of equol from soymilk isoflavones it is better to use bacterial co-culture and enrich soymilk with inulin.

Declarations

Author contribution statement

Salma Elghali Mustafa: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Shuhaimi Mustafa, Amin Ismail, Faridah Abas, Mohd Yaizd ABD Manap: Contributed reagents, materials, analysis tools or data.

Omer. A. A. Hamdi, Salma Elzen, Lutfun Nahar, Satyajit D. Sarker: Analyzed and interpreted the data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

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