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Mustafa, SE, Mustafa, S, Ismail, A, Abas, F, Manap, MYABD, Hamdi, OAA, Elzen, S, Nahar, L and Sarker, SD (2020) Impact of Prebiotics on Equol Production from Soymilk Isoflavones by Two Bifidobacterium species. Heliyon, 6 (10). ISSN 2405-8440

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Research article

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

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A R T I C L E   I N F O

Keywords:
Food science
*Bifidobacterium* spp
Prebiotic
β-Glucosidase
Isoflavones
Transformation

A B S T R A C T

The influence of commercial prebiotics (fructo-oligosaccharides and inulin) and sugars (glucose and sucrose) on enhancing equol production from soymilk isoflavones by *Bifidobacterium longum* BBS536 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; Pee 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-hydroxy-phenyl)-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-isoavandiol) 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 (Raff, 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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https://doi.org/10.1016/j.heliyon.2020.e05298
Received 20 December 2019; Revised in revised form 20 May 2020; Accepted 15 October 2020
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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). Soy milk helps on delivering probiotic to the consumer (Otieno et al., 2005). Moreover, studies reported that, soy milk 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 (Li, 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 soy milk). 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 Bifidobacterium 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 soy milk with prebiotic could enhance bacterial growth in soy milk 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) (Fiorewska 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 equal 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 (Yukeskag et al., 2017; Otieno et 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 equal 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 15700 and 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

Soy milk 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). Soy milk 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
soymilk (5% v/v) batches of and incubated for 48 h at 37 °C. Fifty mil-
liters 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 hy-
drolysis of the substrate p-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 p-NPG (5 mM) was added to 10 ml of each aliquot
and incubated at 37 °C for 30 min (Otzen et al., 2006; Scalabarini 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 cen-
trifuge (Model 5810 R). The quantity of β-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 released 1 μmol of β-nitro-phenol
from the substrate p Namel for 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 (Jiyeon
Desk Top, Seoul, South Korea) and an electronic stirrer (Gas-Col Ltd,
Supelco, Sigma-Aldrich Co. LLC. L, USA), diode array ultravi-
obles investigation by high performance liquid chromatography (HPLC)
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 μm) (Ascenti–Supelco, Sigma-Aldrich Co. LLC. L, USA), diode array ultravi-
obles liquid chromatography (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 me-
tabolites 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 equal production from fermented soymilk. They were: glucose (≥99.5%) and sucrose (≥99.5%) purity [Sigma, Louis, USA], inulin and
fructo-oligosaccharides (Oratlif Pty. Ltd, Tienen, Belgium). The inulin
was used was Rafiline ST with a purity of 92% and an average degree of
polymerization (DP) of 10. The fructo-oligosaccharide (FOS) which util-
ized 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 signifi-
cant differences tests (LSD). Fisher test was used to classify the significant
differences among mean values (P ≤ 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 pop-
ulation 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 Bifi-
dobacteria, 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. acidophilus L10. However,
the cell growth in soymilk fermentation is influenced by the cultures and
fermentation period (Jiyeon et al., 2006).

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
tested organisms. Moreover, there was a significant difference (P ≤ 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 reliable on time and strain. It is noticed that soymilk fermented with B. breve, which had the maximum β-glucosidase activity (4.126 U) at 36 h of fermentation, represented the highest number cell (8.55 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. 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. 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. 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.
The 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* BB536 for 48 h at 37 °C.

### Table 4

| Bacteria species | Sugars | Glucose |
|------------------|--------|---------|
|                  | Daidzin | Daidzein | Equol | Daidzin | Daidzein | Equol |
| *B. breve* | 12 | 10.22 ± 0.02a | 7.54 ± 0.02a | 2.40 ± 0.01a | 10.54 ± 0.02b | 6.40 ± 0.01a | 3.22 ± 0.02b |
| 24 | 8.34 ± 0.02b | 8.61 ± 0.02b | 2.84 ± 0.01b | 9.61 ± 0.02b | 6.84 ± 0.01b | 3.34 ± 0.02b |
| 36 | 6.45 ± 0.03c | 6.37 ± 0.02c | 3.59 ± 0.01c | 8.97 ± 0.02c | 5.59 ± 0.01c | 3.45 ± 0.03c |
| 48 | 5.58 ± 0.03d | 5.94 ± 0.03d | 4.11 ± 0.02d | 8.14 ± 0.03d | 3.81 ± 0.02d | 3.58 ± 0.03d |
| *B. longum* | 12 | 10.17 ± 0.01a | 6.14 ± 0.04a | 2.74 ± 0.02a | 10.44 ± 0.04b | 6.74 ± 0.02a | 2.17 ± 0.01b |
| 24 | 9.45 ± 0.05b | 7.63 ± 0.05b | 2.95 ± 0.11b | 9.83 ± 0.05c | 5.95 ± 0.11b | 3.45 ± 0.05c |
| 36 | 7.46 ± 0.05c | 6.37 ± 0.06c | 3.39 ± 0.05c | 9.37 ± 0.06c | 3.39 ± 0.05c | 3.46 ± 0.05c |
| 48 | 5.48 ± 0.07d | 5.98 ± 0.04d | 3.88 ± 0.06d | 8.68 ± 0.04d | 2.88 ± 0.06d | 3.48 ± 0.07d |
| *B. breve + B. longum* | 12 | 7.26 ± 0.025e | 8.44 ± 0.035e | 3.32 ± 0.01e | 10.14 ± 0.055e | 7.72 ± 0.025e | 4.2 ± 0.0255e |
| 24 | 6.32 ± 0.067h | 9.50 ± 0.023h | 5.97 ± 0.03h | 8.50 ± 0.02h | 6.97 ± 0.03h | 5.32 ± 0.07h |
| 36 | 5.10 ± 0.017i | 6.78 ± 0.043i | 6.61 ± 0.03i | 7.78 ± 0.047i | 5.61 ± 0.037i | 5.90 ± 0.027i |
| 48 | 4.33 ± 0.035e | 3.63 ± 0.029e | 7.31 ± 0.06e | 6.63 ± 0.036e | 4.31 ± 0.06e | 6.33 ± 0.046e |

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 ≤ 0.05).

### 4.4. Effect of prebiotics on equal production

In the current research, the effects of the selected prebiotics such as (inulin, FOS) and glucose and sucrose on equal production from soymilk isolavones 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. breve* BB536 and *B. breve* ATCC 15700. There was noticeable decrease in isoflavone glycoside (daidzin) and daidzein parallel to increasing of equal production by fermentation time.

Table 5 represents the influence of adding sucrose to soymilk on equal production. As shown, by 48 h of incubation, *B. longum* BB536 and *B. breve* ATCC 15700 co-culture delivered high quantity of equal (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 isolavones aglycones and equal production using five strains of isolavones metabolizing microorganism, yielded smaller quantities of aglycones and equal than those observed when soy milk was enriched with fructose and lactose sugars. Results for the effect of glucose addition on soy milk 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 equal 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 equal 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 equal production is varying within the Bifidobacteria species (Table 5). *B. breve* ATCC 15700 showed high amount (4.94 mmol/l) of equal 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 equal 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 isolavone conjugate to produce the correspondence aglycones and equal can be stimulated by FOS. The present results also agree with the finding that addition of FOS to soymilk professionally and significantly (P ≤ 0.05) increases the β-glucosidase activity, and this was
dominant in soymilk fermented with *L. acidophilus* (Yeo and Liong 2010) and with Ohita et al. (2002) who reported FOS enhanced cecal β-glucosidase action and daidzein conversion to equol in both O VX and SH mice. Consequently, these findings 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 daidzein glucoside via β-glucosidase hydrolysis to aglycones than a single bacterial culture. Also it may offer nutrients and circumstances that somehow 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.

#### Funding statement

This work was supported by the Malaysian Government.

#### Competing interest statement

The authors declare no conflict of interest.

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

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 < 0.05). SM = Soy milk.
Additional information

No additional information is available for this paper.

Acknowledgements

We are grateful to Malaysian Government for finance intended for this research.

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