Ann Microbiol (2015) 65:307–319
DOI 10.1007/s13213-014-0863-6

ORIGINAL ARTICLE

Potential ramifications of the effects of sub-lethal ultraviolet B-radiation on the subsequent three subcultures of *Lactobacillus fermentum* BT 8219 during fermentation in biotin-supplemented soymilk and their probiotic properties

Joo-Ann Ewe · Jia-Sin Ong · Wan-Nadiah Wan-Abdullah · Abdul Karim Alias · Min-Tze Liong

Received: 18 December 2013 / Accepted: 28 February 2014 / Published online: 5 April 2014
© Springer-Verlag Berlin Heidelberg and the University of Milan 2014

Abstract The aim of our study was to evaluate the potential ramifications of sub-lethal ultraviolet B radiation (SUVBR) on *Lactobacillus fermentum* BT 8219 cells through to three subsequent subcultures during fermentation in biotin-supplemented soymilk (biotin-soymilk). *Lactobacillus fermentum* BT 8219 cultures were irradiated in Petri dishes (diameter 56 cm^2) with UVB at a dosage of 60 J m^{-2} using a GS Gene Linker UV Chamber. Cell lethality was observed immediately after SUVBR, followed by higher growth than that shown by the control during fermentation in biotin-soymilk ($P<0.05$). This enhanced growth was associated with enhanced β-glucosidase specific activity, leading to increased bioconversion of isoflavone glucosides to aglycones ($P<0.05$). The first, second and third subcultures of the treated cells and the control showed similar growing characteristics, enzymes and isoflavone bioconversion activities in biotin-soymilk ($P>0.05$). In comparison to the control, SUVBR affected the functional properties of parent cells by reducing their tolerability towards acid (pH 2) and bile, lowering their inhibitory activities against selected pathogens and reducing their ability for adhesion ($P<0.05$). Acid tolerance and adhesion ability of the subsequent subcultures of the treated cells showed comparable traits with the control ($P>0.05$), while reduced bile tolerance and antimicrobial property were observed up to the second subcultures of the treated cells ($P<0.05$). Our results suggest that SUVBR could be utilised to produce putative probiotic cells and to increase the bioactivity of biotin-soymilk via fermentation with *L. fermentum* BT 8219 for the development of functional foods.

J.-A. Ewe · J.-S. Ong · W.-N. Wan-Abdullah · A. K. Alias · M.-T. Liong (✉)
School of Industrial Technology, Universiti Sains Malaysia, 11800 Penang, Malaysia
e-mail: mintze.liong@usm.my

Keywords Sub-lethal · UVB · *Lactobacillus fermentum* · Biotin-soymilk · Isoflavones · Probiotic properties

Introduction

The virtues of soymilk have always been accredited to its content of high-quality soy protein, as well as isoflavones, bioactive compounds that have been extensively associated with the prevention of atherosclerosis, cancer, osteoporosis and menopausal disorders (Larkin et al. 2008). Isoflavones are plant-derived phytoestrogens with a structural homology similar to human oestrogens. There are two chemical forms of isoflavones in soy: glucosides (malonyl, acetyl and underivatised glucosides), which are the predominant form, and the bioactive aglycones, which account for only a small proportion (Wei et al. 2007). The chemical structures of the isoflavones and its metabolites influence the extent of absorption, with the aglycone form being more readily absorbed and bioavailable than the highly polar, conjugated glucosides (Kano et al. 2006). Setchell et al. (2002) reported that intestinal microbiota possessing β-glucosidases, such as lactobacilli, are involved in the hydrolysis of the glucose moiety from glucose-conjugated isoflavones, releasing the biologically active aglycone configurations. In a subsequent investigation, Ewe et al. (2011) reported that soymilk fermented with lactobacilli possessing β-glucosidases contains higher

amounts of aglycones than of glucoside conjugates of isoflavones.

In addition to the enhancement of aglycone content, the growth of lactobacilli in soymilk further promotes the product value by releasing organic acids and vitamins, which are beneficial to health, during fermentation (Ewe et al. 2010). We have previously demonstrated that the supplementation of soymilk with B-vitamin, particularly biotin, significantly promotes the growth characteristics and β-glucosidase enzyme activity of the lactobacilli in soymilk, which subsequently enhances the bioconversion of isoflavone glucosides to aglycones during the fermentation of soymilk (Ewe et al. 2010, 2011). Lactic acid bacteria require biotin as an essential micronutrient for normal cellular function, growth and development (Xu et al. 2008). Specifically, biotin is needed as a cofactor for acetyl-CoA carboxylase, which catalyses the first committed step of fatty acid biosynthesis, thereby constituting the building blocks of cellular membrane phospholipid (Cronan and Waldrop 2002), and as a prosthetic group in pyruvate carboxylase involved in anaplerotic metabolism (Davis et al. 2000).

Ultraviolet (UV)-radiation covers the non-ionising region of the electromagnetic spectrum and is intermediate between the X-ray and visible light (200–400 nm) regions. The UV region is categorised into three regions—UVA (320–400 nm), UVB (280–320 nm) and UVC (200–280 nm). UV-radiation causes impairment of cellular functions by generating reactive oxygen species which react with lipids, proteins and nucleic acid and lead to oxidative stress condition. Although UV exposure results in DNA lesions, bacteria have evolved diverse protection mechanisms against UV stress via the production of photo-protective substances or they possess efficient repair processes (Nagira et al. 2002). One adaptive response of bacterial cells to UV-radiation is delay cell growth; for example, Berney et al. (2007) observed a period of lag time before the culture resumed growth after the radiation was removed. In addition, cells that survive a sub-lethal dose of physical treatment will also adapt, thus improving their subsequent performance dramatically in a compromising environment; this property is particularly significant in biotechnology applications (Prasad et al. 2003). We have previously demonstrated that sub-lethal UV B radiation (SUVBR) significantly enhances cell growth and β-glucosidase enzyme and isoflavone bioconversion activities of *L. fermentum* BT 8219 in biotin-enhanced/supplemented soymilk (biotin-soymilk), mainly via alteration of the cellular membrane (Ewe et al. 2013). However, the potential ramifications of such a treatment on the subsequent subcultures of cells remain unknown.

Probiotics have been defined by the Food and Agriculture Organisation/World Health Organisation as living microorganisms that are believed to confer a health benefit to the host when consumed in adequate amounts. Many strains of lactobacilli are considered to have with probiotic properties, with health effects ranging from the modulation of lactose intolerance and the lipid profile to the alleviation of post-menopausal disorders (Liong et al. 2007). Many criteria have to be fulfilled in order for a microbial strain to be considered a probiotic, such as a lack of pathogenicity, tolerance of gastro-intestinal conditions (acid and bile), ability to adhere to the gastrointestinal mucosa and the competitive exclusion of pathogens (Liong and Shah 2005). The exposure of cells to external stimulation, such as SUVBR, could alter the membrane surface of bacterial cells and consequently induce changes in the physico-chemical properties of these cells (Schär-Zammaretti and Ubbink 2003). In lactobacteria considered to be promising probiotics, such modifications may affect the probiotic properties, which are closely related to cellular integrity and the structure of the bacterial cell surface (Golowczyc et al. 2011). To date, no study has evaluated the probiotic properties of SUVBR-treated lactobacilli and the sustainability of the treatment in affecting the probiotic properties for a few subsequent subcultures. These unknown factors should therefore be clearly elucidated.

The aim of this study was to investigate the effects of SUVBR on the growth of *Lactobacillus fermentum* BT 8219 and its isoflavone bioconversion ability, as well as the potential ramifications of the treatment being passed on to subsequent subcultures of cells during fermentation in biotin-supplemented soymilk (biotin-soymilk). The effects of SUVBR on the probiotic properties of *L. fermentum* BT 8219 were also evaluated.

Materials and methods

Bacterial cultures

Lactobacillus fermentum BT 8219 and the indicator organisms for the antimicrobial activity assay (probiotic properties), such as *Escherichia coli* USM 4144, *Staphylococcus aureus* USM 4144, *Salmonella typhimurium* USM 4144 and *Klebsiella pneumonia* USM 4144, were obtained from the Culture Collection Centre of Food Technology Division, School of Industrial Technology, Universiti Sains Malaysia (Penang, Malaysia). The *L. fermentum* BT 8219 and the pathogens were activated from stock cultures [stored at −20 °C in 40 % (v/v) sterile glycerol] in sterile MRS broth and tryptic soy broth (Hi Media, Mumbai, India), respectively, in three consecutive cultures prior to experimental use.

Preparation of biotin-soymilk

The biotin-soymilk was prepared using methods previously described (Ewe et al. 2013). Briefly, soaked soybeans were

blended with distilled water at a ratio of 1:6 (w/v). The resultant slurry was then filtered to obtain soymilk, which was pasteurised at 63 °C for 30 min and then cooled immediately to 4 °C. Upon cooling, filter-sterilised biotin (Sigma-Aldrich, St. Louis, MO) was added to soymilk to a final concentration of 1 mg L^{-1}.

SLUVB radiation of *L. fermentum* BT 8219 and propagation of cultures

Activated *L. fermentum* cells were harvested from MRS broth by centrifugation at 3,500 *g* for 10 min at 4 °C. The cell pellets collected were washed twice and re-suspended in sterile phosphate-buffered saline (PBS, pH 7). The cell suspensions (10 %, v/v) were irradiated in Petri dishes (diameter 56 cm^2) with UVB at a dosage of 60 J m^{-2} using a GS Gene Linker UV Chamber (Bio-Rad Laboratories, Hercules, CA). The treated lactobacilli (with non-treated cells as a control) was used as the parent cultures. The parent cells were then transferred into fresh biotin-soymilk (10 %, v/v) level and fermented for one growth cycle at 37 °C for 24 h to generate cultures of the first subculture (Birošová and Mikulášová 2009). Subsequent propagation was carried out to produce second and third subcultures, which involved utilisation of cultures (10 %, v/v) from the previous subculture followed by incubation at 37 °C for 24 h. Cultures from each subculture (parent, first, second and third subcultures with a corresponding control for each subculture) were subjected to analyses of their functional properties and inoculated (5 %, v/v) into biotin-soymilk for fermentation.

Fermentation of *L. fermentum* BT 8219 in biotin-supplemented soymilk

Fermentation of control and SUVB-treated cells of *L. fermentum* BT 8219 in biotin-supplemented soymilk were performed at 37 °C for 24 h. The samples were withdrawn aseptically from the fermented soymilk at 4 h interval for enumeration of viable counts, determination of intracellular and extracellular β-glucosidase enzyme activity and detection of isoflavone concentrations.

Growth of *L. fermentum* BT 8219 in biotin-soymilk

Both control and SUVB-treated lactobacilli were cultured in biotin-soymilk for 24 h. The cultures were sampled at 4-h interval and cultured using the pour plate method on Petri plates containing MRS agar at 37 °C for 48–72 h. Viable counts were recorded as colony-forming units (CFU) per millilitre of the biotin-soymilk.

Assay for intracellular and extracellular β-glucosidase activities

The intracellular and extracellular β-glucosidase activity of control and SUVB-treated *L. fermentum* was assayed using methods previously described (Ewe et al. 2013). For the intracellular β-glucosidase assay, the bacterial cells were sonicated in an ice-bath for 30 min, and the cell suspension was extracted in a cold sodium citrate buffer (50 mmol L^{-1}, pH 5.5). The cellular extract thus obtained was used for analysis as the crude enzyme extract in a reaction with *p*NPG (*p*-nitrophenyl-β-D-glucopyranoside) during incubation at 37ºC for 30 min. The amount of *p*-nitrophenol released from the reaction was measured at 420 nm to determine the intracellular β-glucosidase activity. One unit of enzyme activity was defined as the amount of enzyme that released 1 μmol of *p*-nitrophenol from *p*NPG per millilitre per minute under assay conditions. The protein concentration of the crude enzyme extract was determined using the Bradford (1976) method with bovine serum albumin as the standard (Sigma, St. Louis, MO). Specific activity was expressed as milli-units (mU) of β-glucosidase activity per milligram of protein.

The extracellular β-glucosidase activity of the lactobacilli culture was determined by measuring the rate of hydrolysis of *p*NPG. The amount of *p*-nitrophenol released was determined by the same method as described for intracellular β-glucosidase activity.

Evaluation of bioconversion of isoflavones in biotin-soymilk

The isoflavones from fermented biotin-soymilk were extracted and analysed as described in a previous study (Ewe et al. 2013). Concentrations of isoflavones were determined using high-performance liquid chromatography (HPLC). The HPLC system was equipped with a UV-2077 Plus, 4-λ Intelligent UV/Vis Detector (Jasco, Tokyo, Japan) that was set at 259 nm fitted with an Inertsil ODS-3 column (150× 3 mm, 5 μm; GL Sciences, Tokyo, Japan) that was maintained at 40 °C. Samples were eluted at a flow rate of 1 mL min^{-1} using a mobile phase that consisted of solvent A (water:phosphoric acid, 1,000:1, v/v) and solvent B (water:acetonitrile:phosphoric acid, 200:800:1, v/v/v). Gradient elution was used to isolate the isoflavones and was set as follows: solvent A 100 % (2 min)→65 % (29 min)→ 50 % (31 min)→100 % (40 min)→100 % (43 min). HPLC-grade glucosides and aglycones were used as standards.

Evaluation of probiotic properties

Acid tolerance

The acid tolerance of *L. fermentum* BT 8219 was determined according to the method described by Teh et al. (2009).

Briefly, *L. fermentum* BT 8219 (10 %, v/v) was inoculated into pepsin-supplemented MRS broth cultures that were adjusted to pH 2.0 and 3.0, respectively, with HCl and incubated at 37 °C for 3 h. Viable bacteria in the MRS broth cultures were enumerated by taking samples every 30 min for 3 h, subsequently plating 1 mL of a serially diluted sample on MRS agar plates and incubating the plates at 37 °C for 48–72 h. Acid tolerance was determined by comparing the final plate count after 3 h with the initial plate count at 0 h.

Bile tolerance

The bile tolerance of *L. fermentum* BT 8219 was determined against oxgall, cholic acid and taurocholic acid (Sigma-Aldrich) according to the method described by Teh et al. (2009). MRS broth containing 0.3 % (w/v) oxgall, cholic acid or taurocholic acid was inoculated with lactobacilli and incubated at 37 °C. The control consisted of MRS broth without bile salt. Bacterial growth was measured at hourly intervals for 7 h with a spectrophotometer (Shimadzu, Kyoto, Japan) at 620 nm. The absorbance values obtained were plotted against incubation time, and the bile tolerance of lactobacilli was considered to be the time required for the absorbance value to increase by 0.3 units.

Antimicrobial activity assay

The antimicrobial activities of *L. fermentum* BT 8219 on test pathogens, such as *Escherichia coli*, *Staphylococcus aureus*, *Salmonella typhi* and *Klebsiella pneumonia* were evaluated by the disc diffusion assay (Blažeka et al. 1991). The activated test pathogens with a turbidity of 0.3 ($OD_{600\ nm}$) were seeded on tryptone soy agar (Hi Media), following which sterile filter paper discs (diameter 5 mm) containing 20 μL of *L. fermentum* BT 8219 cultures were placed on the surface of the agar media. The plates were incubated at 37 °C for 24 h, and the diameter of the zones of inhibition (mm) was measured.

In vitro adhesion to mucin

The adhesion ability of *L. fermentum* BT 8219 to mucin was examined by the method of Azcarate-Peril et al. (2009). The partially purified type III porcine gastric mucin (100 μL of 10 mg mL^{-1}; Sigma) was immobilised in 96-well microtiter plates by incubation at 4 °C for 24 h. Excessive mucin was removed by pipetting, and wells were washed twice with 200 μL of PBS.

A 100-μL sample of *L. fermentum* cultures was added to each well and the plates incubated for 3 h at 37 °C, followed by seven separate washes with 200 μL PBS to remove any unbound bacteria. Triton-X-100 solution (200 μL; 0.05 %, v/v) was then added to each well to treat and desorb the bound bacteria. A 100-μL sample of the suspension of each well was removed and tenfold serial dilutions made using 0.9 % (w/v) NaCl before plating the diluted samples onto MRS agar. The plates were incubated at 37 °C for 48–72 h.

Statistical analysis

Data were evaluated using SPSS ver. 11.5 software (SPSS Inc., Chicago, IL). All data are presented as the mean of three separate runs (n=3). An independent t test was used to determine whether the difference between means was significant at a significance level of α=0.05.

Results and discussion

Growth of *L. fermentum* BT 8219 in biotin-soymilk

Sub-lethal ultraviolet B radiation has been shown to cause physical perturbation of the cytoplasmic and plasma membranes via the formation of reactive oxygen species (ROS). The inability of the injured bacterial cells to repair and recover from the detrimental effects of SUVBR would lead to inactivation of microorganisms (Liu et al. 2010; Ukuku and Geveke 2010). This lethal effect of SUVBR was observed in this study, as evidenced by the reduced viability of lactobacilli (5.6 % compared to the control) immediately following SUVBR (Fig. 1; P<0.05). This reduced cell viability may be due to the effects of ROS generated upon SUVBR which could cause direct damage to key proteins, enzymes and DNA in the cells and indirect oxidative damage to biomolecules (Nagira et al. 2002).

After termination of SUVBR, the UV-treated cells were able to resume growth and multiply in biotin-soymilk within the first 4 h, subsequently exhibiting higher (P<0.05) growth (up to 14.2 % higher) than the control. Previous studies have shown that one of the adaptive responses of bacterial cells to UV is a growth delay, which is a period of lag time before the culture resumes growth after removal of the radiation (Berney et al. 2007). The damaged cellular membranes are repaired during this adaptive period, such as the utilisation of biotin for the regeneration of fatty acid building blocks (Davis et al. 2000). Additionally, it has been suggested that low doses of UVB induce cell protection responses to counteract the toxic effects of UVB oxidative stress (Perluigi et al. 2010). Thus, the SUVBR-induced oxidative stress observed in our study could have caused subtle lipid peroxidation of the cellular membrane; this membrane impairment may then have facilitated the influx of nutrients and oxygen and excretion of wastes from cells, which in turn enhanced their growth.

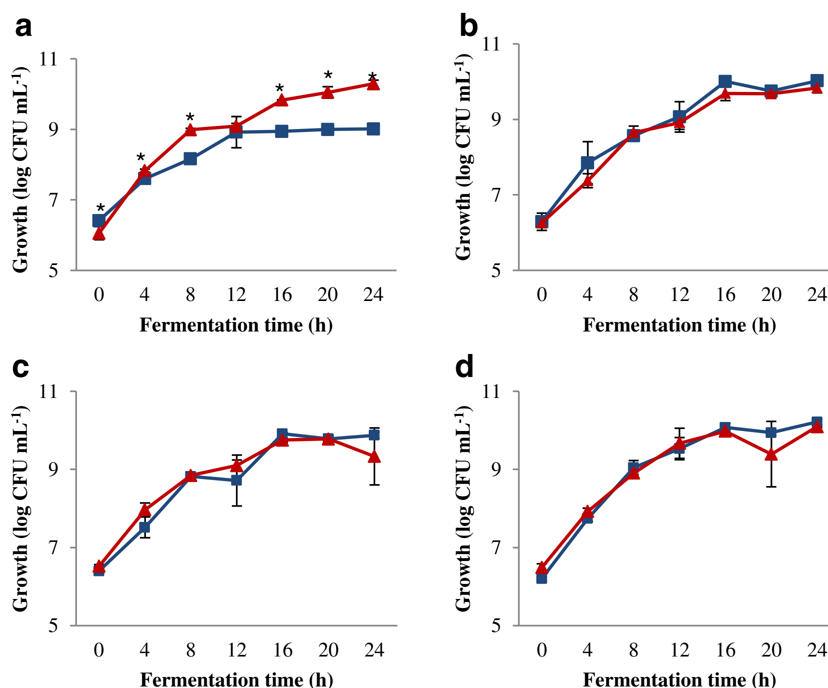

Fig. 1 Growth profiles of control (*blue-shaded squares*) and ultraviolet (UV)-treated (*red-shaded triangles*) *Lactobacillus fermentum* BT 8219 in biotin-supplemented soymilk (biotin-soymilk) during fermentation at 37 °C for 24 h. **a** Parent culture, **b** first subculture, **c** second subculture, **d** third subculture. *Control* Untreated cells; a corresponding control was used for comparison in each subculture. *Error bars* Standard error of the mean (SEM; n=3). *Asterisk* P<0.05 when comparing the control and UV-treated cells in an independent t test

We have previously shown that UV-radiation can induce cell membrane permeability as a result of lipid peroxidation (Ewe et al. 2013). The generation of ROS has been reported to cause oxidative destruction of the cell components through oxidative damage of membrane lipids and proteins (Shiu and Lee 2005). However, bacteria possess self-protection mechanisms against oxidative stress, including enzymes (e.g. catalase and superoxide dismutase), small proteins (e.g. thioredoxin and glutaredoxin) and molecules (e.g. glutathione) (Cabiscol et al. 2000) which allow them not only to successfully adapt to UVB stress but also to survive and grow (He et al. 2002). Given the ability of *Lactobacillus* to release antioxidant compounds and counteract the oxidative stress induced by UV, we investigated the prolonged effects of SUVBR on the growth traits of the affected cells for a few subsequent subcultures in biotin-soymilk. Our results illustrate that the UV-treated cells showed growth during the first, second and third subcultures (P>0.05) that was comparable to that of the control in biotin-soymilk (Fig. 1b–d), suggesting that the treated cells had repaired the damage inflicted by the UV-radiation and recovered from the membrane injury during the propagation process. Various *Lactobacillus* and *Bifidobacterium* species have been shown to scavenge superoxide anion and ROS and thus inhibit lipid peroxidation (Lin and Yen 1999; Lin and Chang 2000). Therefore, the presence of antioxidant enzymes or the release of antioxidant compounds could have assisted in repairing the pores induced by SUVBR. Restoration of the damaged cellular membrane enables the majority of the affected cells to resume their normal physiological activities.

Specific activity of intracellular and extracellular β-glucosidase

Lactobacillus fermentum BT 8219 is known to possess intracellular and extracellular β-glucosidase activities that account for the cleavage of the β-1,6-glycosidic linkage to release bioactive aglycones during the fermentation of soymilk (Ewe et al. 2011). However, effective bioconversion of isoflavones by *L. fermentum* in soymilk is often restricted by the diffusion of substrates/enzymes across the cellular membrane. Thus, in terms of membrane permeability, we assessed UV-treatment, which has been shown to cause physical perturbation of the plasma membrane by increasing membrane permeability to the substrate, as a means to enhance the specific enzyme activity of bacteria.

SUVBR significantly affected both intracellular and extracellular β-glucosidase specific activities of the parent *L. fermentum* culture. The intracellular and extracellular specific enzyme activities of the treated parent cells were 27.0–73.6 % (Fig. 2a) and 20.2–91.3 % (Fig. 3a), respectively, higher (P<0.05) than that of the control. This corresponded well with the higher growth of the treated parent cells than with growth of the control. The higher extracellular enzyme activities may be due to the homeostatic effect of the bacteria to compromise the sudden downshift of osmolarity as a result of membrane reorganisation and upsurge of membrane permeability upon SUVBR (Heppel 1969; Smith et al. 2009). Likewise, the SUVBR-induced membrane permeability may have triggered an elevated influx of substrate isoflavone glucosides intracellularly, leading to a higher synthesis of

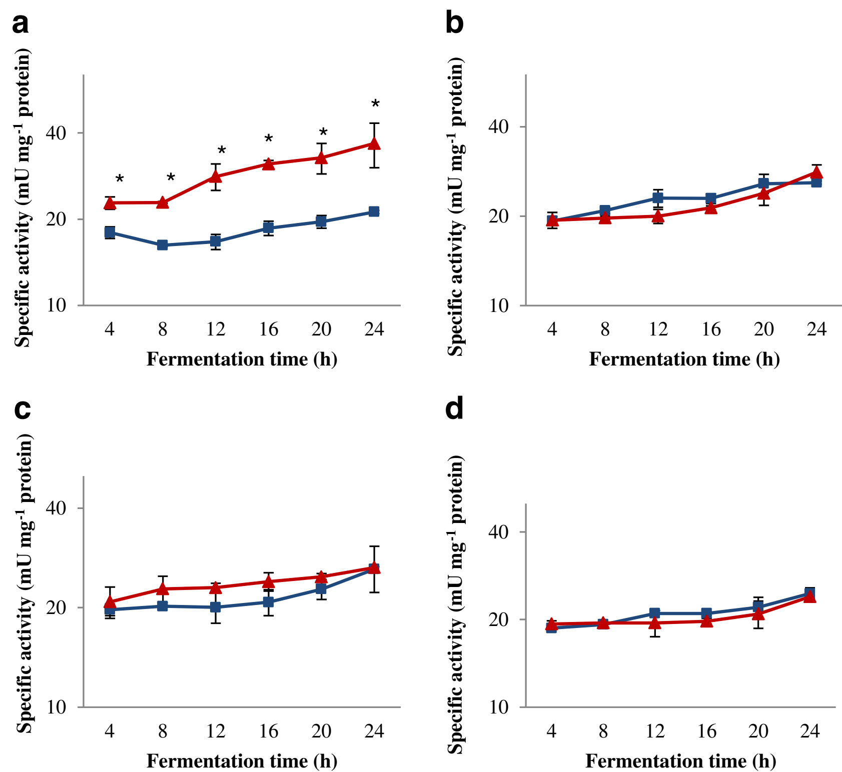

Fig. 2 Intracellular β-glucosidase specific activity of control (*blue-shaded squares*) and UV-treated (*red-shaded triangles*) *L. fermentum* BT 8219 cells in biotin-soymilk during fermentation at 37 °C for 24 h. **a** Parent culture, **b** first subculture, **c** second subculture, **d** third subculture. *Control* Untreated cells; a corresponding control was used for comparison in each subculture. *Error bars* SEM (n=3). *Asterisk* P<0.05 when comparing the control and UV-treated cells in an independent t test

intracellular enzymes for the utilisation of glucose moieties cleaved by the enzymes.

Data from our study also show that UV-treated cells of the first, second and third subcultures and control cells had similar intracellular (Fig. 2b–d) specific activities, possibly due to the initiation of cell protection responses upon the removal of radiation, followed by the repair of the UV-affected membrane lipid bilayer during propagation of the treated parent cells. The restoration of cellular membrane structure enabled the affected cells to regain their regular growth trait. Nevertheless, the

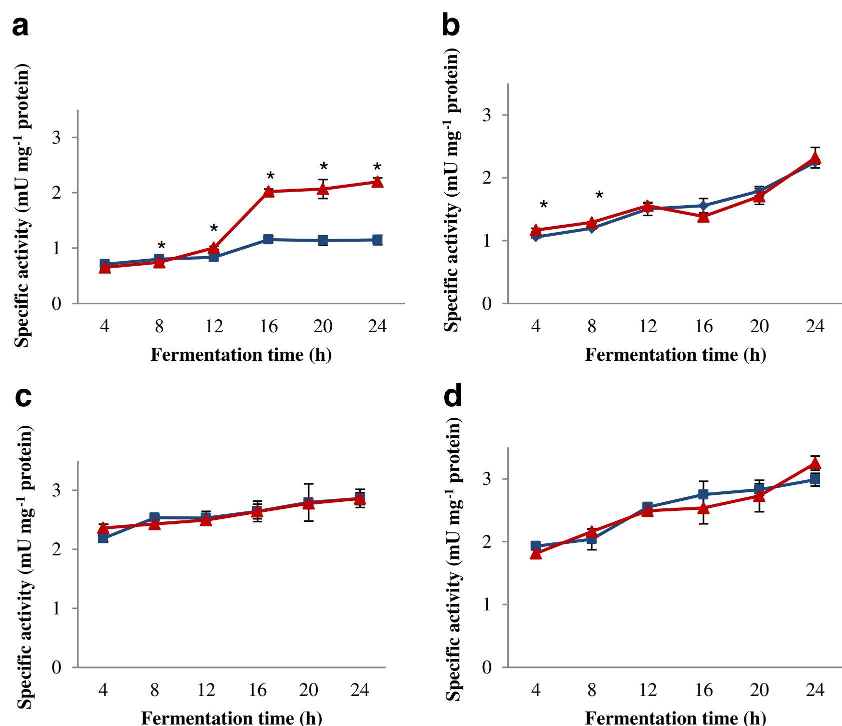

Fig. 3 Extracellular β-glucosidase specific activity of control (*blue-shaded squares*) and UV-treated (*red-shaded triangles*) *L. fermentum* BT 8219 cells in biotin-soymilk during fermentation at 37 °C for 24 h. **a** Parent culture, **b** first subculture, **c** second subculture, **d** third subculture. *Control* Untreated cells; a corresponding control was used for comparison in each subculture. *Error bars* SEM (n=3). *Asterisk* P<0.05 when comparing the control and UV-treated cells in an independent t test

effect of SUVBR was passed on by the treated cells to the first subculture (Fig. 3b) where the treated cells exhibited higher (7.5–10.4 %; $P<0.05$) extracellular specific enzyme activities than the control during the first 8 h of fermentation. However, the effect diminished during the following 12 h up until the end of the fermentation period. Krishnamurthy et al. (2010) demonstrated that UV-radiation induced oxidative stress and enhanced lipid peroxidation, leading to cell-wall damage. He et al. (2002) reported that the repair of lipid peroxidation and reversal of the effects of oxidative stress on the affected cellular membrane induced by UVB radiation was a slow process. Thus, the replacement of lipid in the UVB-radiated cells would need a longer period to resume normal activity. The slow repair of the membrane lipid bilayer may account for the higher extracellular β-glucosidase activity observed in the treated cells during the first 8 h (in the first subculture). Following this time, the affected membrane may have been completely repaired, accounting for the comparable extracellular β-glucosidase specific activity in the control and UV-treated cells of the second and third subcultures during growth in biotin-soymilk (Fig. 3c, d).

Concentrations of isoflavone glucosides and aglycones

The concentrations of isoflavone glucosides (β- and malonyl-derivatives of daidzin, glycitin and genistin) decreased gradually during the fermentation of biotin-soymilk by *L. fermentum* for 24 h; concomittantly, the concentrations of isoflavone aglycones (daidzein, glycitein and genistein) increased (Fig. 4). The decreases in the concentrations of isoflavone glucosides detected in biotin-soymilk upon fermentation by UVB-treated parent cells were greater than those observed in the control cells (reduction of 55.6–82.7 % vs. reduction of 42.0–77.3 %, respectively) (Fig. 4ai, ii and iii). Concurrently, the increases in the concentrations of isoflavone aglycones detected in biotin-soymilk upon fermentation by UVB-treated parent cells were greater than those in the control cells (increase of 155.4–372.4 % vs. increase of only 20.2–137.4 %, respectively). This phenomenon correlated well with the increased intracellular and extracellular β-glucosidase specific activity determined during the growth of UV-radiated cells in biotin-soymilk. Mahajan et al. (2012) also suggested that a physical treatment-induced permeabilised membrane could expose the active sites of membrane-bound enzymes to substrates and lead to enhanced bioconversion of isoflavones. Clinical studies have revealed that aglycones, such as daidzein and genistein, could improve the biological functions of the human body, such as lowering the risk of coronary artery disease and reducing post-menopausal symptoms (Ren et al. 2001). Thus, increased concentrations of aglycones in soymilk are often desirable as aglycones are absorbed faster and in greater amounts than glucosides. The changes in the concentrations of isoflavones in biotin-soymilk during fermentation by treated cells of the first, second and third subcultures were comparable to those of of the control (Fig. 4b–d). These similar trends in the bioconversion of isoflavones correlated well with the growth and enzyme activities of the cells in biotin-soymilk as a result of comparable growth trait of treated cells with that of the untreated cells. We found that UV-radiation was able to enhance membrane permeabilisation as a consequence of lipid peroxidation, followed by resealing of the membrane upon the removal of treatment, as in our previous study (Ewe et al. 2013). This indicated that the injured membrane lipid bilayer was completely repaired during propagation of the treated parent cells and that the cell had resumed normal growth.

Acid tolerance

The ability of putative probiotic *L. fermentum* BT 8219 cells to survive passage through the gastrointestinal tract is mainly attributed to their acid tolerance ability (Kirjavainen et al. 1998). In our study, *L. fermentum* exhibited tolerance to low pH conditions (pH 2 and 3), as evidenced by the high viabilities of control and UV-treated parent cells (>5 $\log_{10}$ CFU mL^{-1}) after 3 h of incubation (Fig. 5a). UV-radiation did not affect the acid tolerance of *L. fermentum* cultured at pH 3—both treated and control parent cells were highly tolerant. Nonetheless, the resistance of the treated parent cells to acidity at pH 2 was 4.4–18.4 % ($P<0.05$) lower than that of the control. The acid tolerance ability of Gram-positive cells has been reported to be due to such mechanisms as proton pumping, changes in the cell membrane and regulatory mechanisms (Cotter and Hill 2003). Thus, the decrease in the acid resistance ability of *L. fermentum* may be due to alterations in the membrane-bound enzyme (ATPase) that is responsible for translocating H^+ ions across the membrane of microorganisms (Hong et al. 1999) for the regulation of cytoplasmic pH by UV-radiation. We have previously shown that UV-radiation targets alterations in the membrane lipid bilayer by inducing oxidative stress (Ewe et al. 2013) and that these alterations may have subsequently altered the protein structure of ATPase. The high proton motive force of pH 2 could have caused severe membrane damage to the membrane-injured UV-treated parent cells, leading to the loss of viability and activity under the condition of severe acidification (Teh et al. 2009). However, the acid resistance characteristic of control and treated cells of the first, second and third subcultures (Fig. 5b–d) were similar, suggesting that the injured membrane lipid bilayer was very well repaired during propagation.

Bile tolerance

The resistance of *L. fermentum* BT 8219 cells to simulated gastric content was used as a marker of tolerance to bile acids (Schillinger et al. 2005). Our results showed that *L. fermentum*

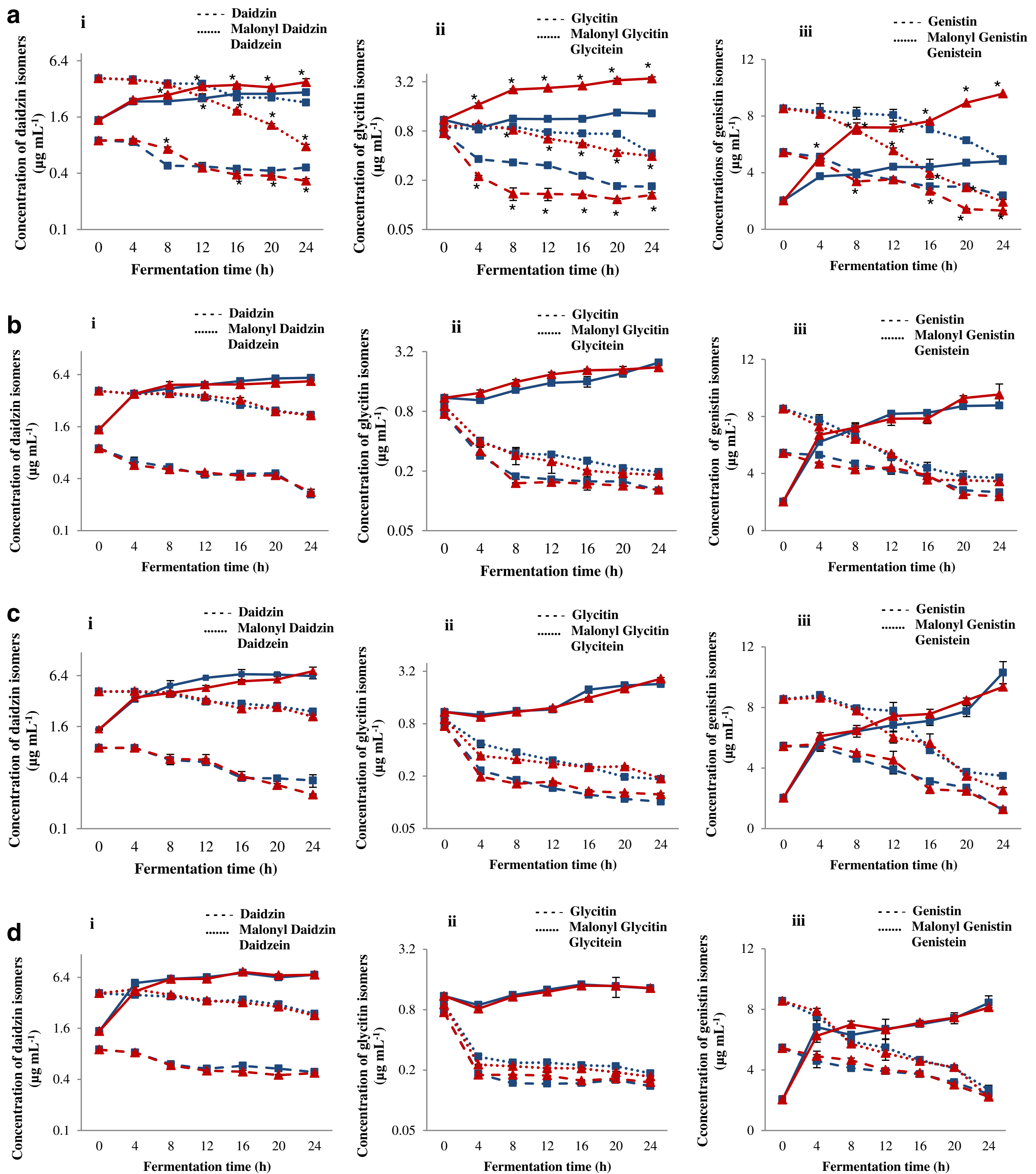

Fig. 4 Changes in the concentration of daidzin conjugates (*I*), glycitin conjugates (*ii*) and genistin conjugates (*iii*) in biotin-soymilk fermented by control (*blue-shaded squares*) and UV-treated (*red-shaded triangles*) *L. fermentum* BT 8219 cells at 37 °C for 24 h. **a** Parent cells, **b** first subculture, **c** second subculture, **d** third subculture. *Control* Untreated cells; a corresponding control was used for comparison in each subculture. *Error bars* SEM (n=3). *Asterisk* P<0.05 when comparing the control and UV-treated cells in an independent t test

BT 8219 cells were more sensitive to cholic acid (deconjugated bile) than to taurocholic acid and oxgall (Fig. 6). Bile acid is considered to be one of the more deleterious stress compounds that inhibit the growth of intestinal bacteria through their membrane-damaging effect. The hydrophobicity of deconjugated bile acids gives rise to their

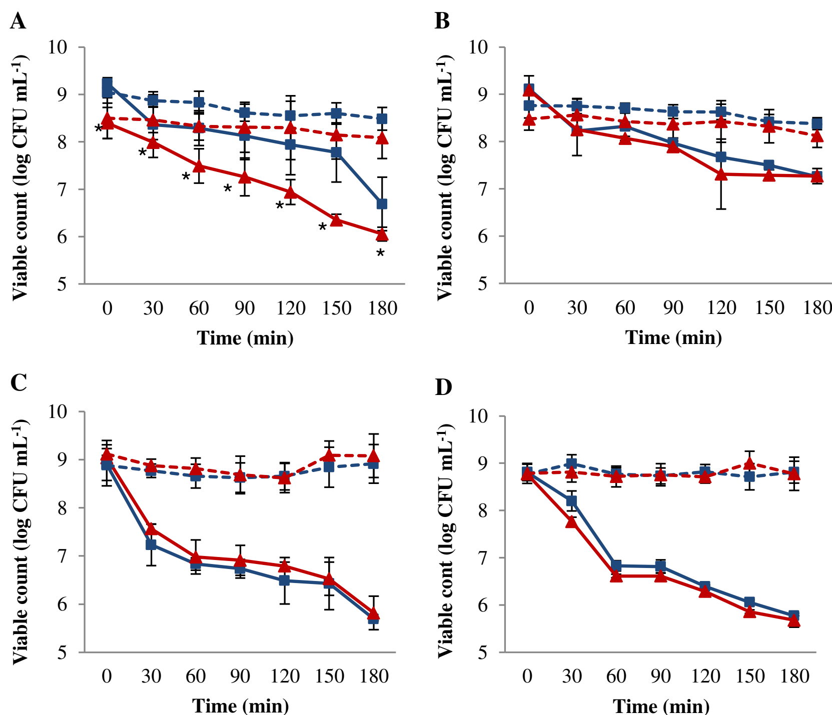

Fig. 5 Effect of pH 2.0 (*solid line*) and 3.0 (*dotted line*) on the viability of control (*blue-shaded squares*) and UV-treated (*red-shaded triangles*) *L. fermentum* BT 8219 cells at 37 °C for 180 min. **a** Parent cells, **b** first subculture, **c** second subculture, **d** third subculture. *Control* Untreated cells; a corresponding control was used for comparison in each subculture. *Error bars* SEM (n=3). *Asterisk* $P<0.05$ when comparing the control and UV-treated cells in an independent t test

cellular damaging effects, and thus they have greater inhibitory effects against bacterial cells than their conjugated counterparts (Yokota et al. 2000). The similar tolerability of *L. fermentum* to conjugated taurocholic acid and oxgall has been attributed to the higher proportion of conjugated bile acids in oxgall (Elkins and Mullis 2004).

The bile tolerance of UV-treated parent cells to oxgall, cholic acid and taurocholic acid was 104.3, 12.3 and 98.4 % lower, respectively than that of the control (Fig. 6a; $P<0.05$). It has been suggested that the stability of the *Lactobacillus* lipid membrane plays a role in the bile tolerance characteristics of cells (Murga et al. 1999; Kimoto et al. 2002). UV-radiation has been reported to cause the formation of free radicals and ROS (Herrling et al. 2003) that attack the unsaturated fatty acids of the cellular membrane, leading to lipid peroxidation and changes in the lipid bilayer, as demonstrated

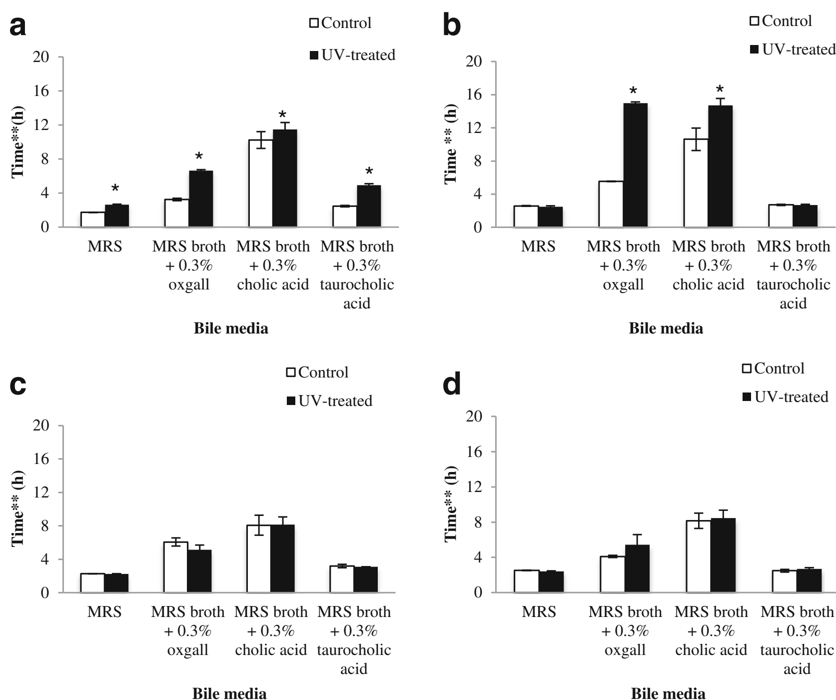

Fig. 6 Bile tolerability of control and UV-treated *L. fermentum* BT 8219 cells in different bile media. **a** Parent cells, **b** first subculture, **c** second subculture, **d** third subculture. *Control* Untreated cells; a corresponding control was used for comparison in each subculture. Results are expressed as the mean ± SEM (n=3). *Asterisk* indicates significant difference in the means within the same type of bile media between the control and UV-treated cells ($P<0.05$) in an independent t test. *Time*** Time (h) required to increase absorbance by 0.3 units at 620 nm

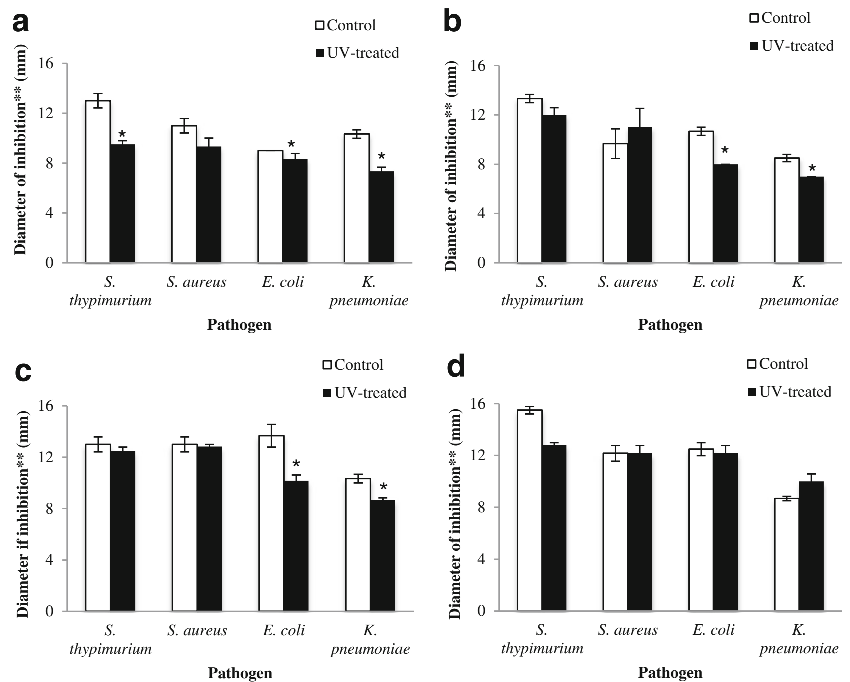

Fig. 7 The antimicrobial activities of *L. fermentum* BT 8219 on test pathogens were evaluated by the disc diffusion assay in which discs impregnated with 20 μL *L. fermentum* BT 8219 culture were placed on agar seeded with the test pathogens. The diameter of the area of clearing surrounding the disc, referred to as the zone of inhibition, was used as an indirect indicator of the inhibition ability of *L. fermentum* BT 8219, i.e., the larger the diameter of zone of inhibition, the greater the inhibition

by Ewe et al. (2013). Thus, exposure of the non-stable lipid membrane of UV-treated parent cells to the membrane-damaging bile acids may be one factor contributing to the lower tolerance of the treated cells compared to the control.

The bile tolerability of UV-treated cells persisted to the first subculture (Fig. 6b; $P<0.05$), during which the treated cells showed 169.2 and 38.48 % lower tolerance towards oxgall and cholic acid, respectively, than the control. This lower tolerance may be due to the restoration of the UV-permeabilised membrane requiring energy and the synthesis of lipid (He et al. 2002); consequently, a longer time was needed for the cell to restore normal membrane structure despite the rearrangements which occurred in the phospholipid bilayer immediately after treatment. The UV-treated cells of the second and third subculture exhibited a similar bile tolerability to the control (Fig. 6c, d). The restoration of the bile tolerability of cells may be due to their ability to completely repair the injured membrane lipid bilayer.

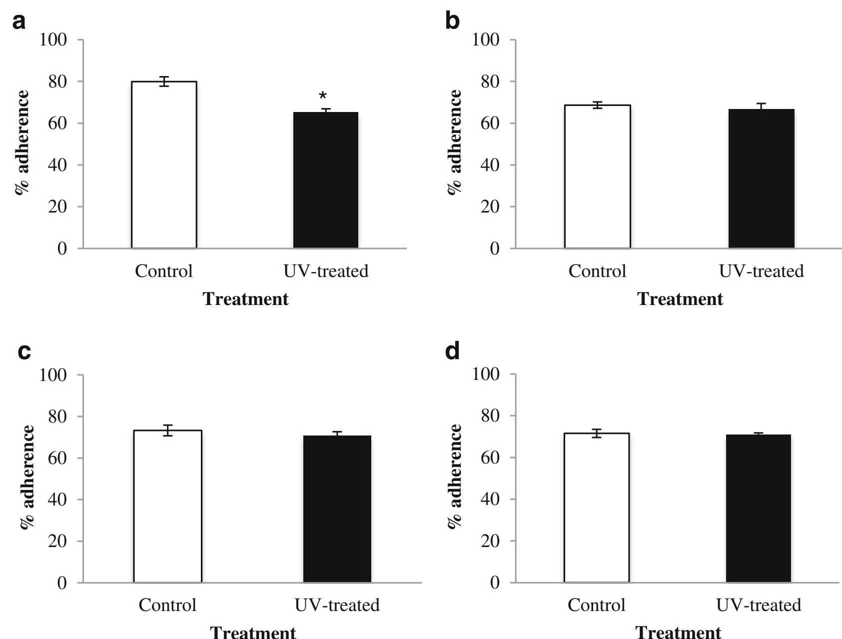

Fig. 8 Adherence abilities of control and UV-treated *L. fermentum* BT 8219 to mucin after incubation for 3 h at 37 °C. **a** Parent cells, **b** first subculture, **c** second subculture, **d** third subculture. *Control* Untreated cells; a corresponding control was used for comparison in each subculture. Results are expressed as the mean ± SEM ($n=3$). *Asterisk* Significant difference between the control and UV-treated cells in an independent *t* test ($P<0.05$)

Antimicrobial activity

The antimicrobial activity of *L. fermentum* BT 8219 is vital for the successful colonisation of lactobacilli in the intestinal mucosa. Lactobacilli have a barrier effect and provide a defence against pathogens via the production of antimicrobial substances (Vaughan et al. 1999). The inhibitory activities of UV-treated parent cells against *Salmonella thypi*, *Escherichia coli* and *Klebsiella pneumoniae* were lower than those of their controls (26.9, 7.4 and 29.0 %, respectively; Fig. 7a; $P<0.05$). The antimicrobial mechanisms of action of *L. fermentum* against the pathogens involved the lowering of the pH due to the production of lactic acid and other metabolites, such as hydrogen peroxide, short-chain fatty acids and bacteriocins such as nisin, reuterin and plantaricin (Šušković et al. 2010). The excretion of these antimicrobial agents across the cytoplasmic membrane is facilitated by a protein transporter that aligns on/across the membrane (Gajic et al. 2003). UVB-radiation has been shown to mediate the generation of ROS and the induction of oxidative damage to lipids and proteins (Baumstark-Khan et al. 2000). Such physical perturbation of the plasma membrane can result in the immediate accumulation of lipid peroxide and a change in membrane fluidity (Ewe et al. 2013). The exposure of protein resides on/in the membrane lipid bilayer to oxidative stress can lead to modifications of amino acid side chains and, consequently, alterations in the protein structure (Cabiscol et al. 2000). Thus, the SUVBR–induced alteration in the carrier protein that caused inefficient secretion of bacteriocins could account for the observed lower inhibitory activities of treated parent cells compared to that of the control.

The antimicrobial activity of UV-treated cells of *L. fermentum* against pathogens was passed on by treated parent cells to the first and second subcultures; the inhibitory activities of these treated cells against *E. coli* and *K. pneumoniae* were 25.0 and 17.7 % (first subculture) and 22.0 and 16.1 % (second subculture) lower, respectively, than those of the untreated cells (Fig. 7b, d). This may due to the need for extended recovery periods by the altered membrane enzymes from being driven into ordinarily improbable conformational states, as a UVB-stressed cell would normally utilise its energy for UV protection mechanisms (Matallana-Surget et al. 2012). Therefore, the incompetent repair of the transport protein during the propagation of treated parent cells could have been passed on to the first and second subcultures, leading to inefficient secretion of bacteriocins extracellularly and thus lower inhibitory activities.

Nevertheless, the antimicrobial activity of UV-treated cells of the third subculture cells of *L. fermentum* against pathogens was different ($P>0.05$), although insignificantly, from that of the control (Fig. 7d). The impaired protein of the treated cells may have recovered and regained its ordinary transport capability, which would thus have enabled the cells to resume their regular inhibitory activity similar to that of the untreated cells.

Adherence ability

The ability of *L. fermentum* BT 8219 to adhere to intestinal mucosa is often a prerequisite for probiotics to colonise the gut in order to exert beneficial health effects (Forestier et al. 2000). UV-treated *L. fermentum* was able to adhere to gastric mucin, although the adherence ability of the parent cells was 14.7 % ($P<0.05$) lower than that of the control (Fig. 8a). Bacterial cell surface hydrophobicity has been reported to be one of the factors affecting cell adhesion. The interactions between *L. fermentum* and intestinal epithelial cells are thus influenced by the composition of the cell surface of the lactobacilli, which consists of proteins, teichoic acids and polysaccharides (Chen 2007). In an earlier study we showed that UV-radiation affected the cell surface of cells by, for example modifying the protein conformation (Ewe et al. 2013), which likely reduced the adherence property of the treated lactobacilli.

Nevertheless, the adherence ability of treated cells of the first, second and third subcultures was equivalent to that of their controls ($P>0.05$) (Fig. 8b–d). This may be due to the treated cells restoring the impaired structures and being able to resume normal adherence ability in their membranes by way of a self-repairing mechanism during propagation. Our results suggest that soymilk fermented by *L. fermentum* BT 8219 may be developed as functional food with probiotic properties. Soymilk fermented by lactic acid bacteria has been reported to result in better sensorial qualities and rheological behaviours in terms of viscous and pseudoplastic properties (Donkor et al. 2007). Also, when compared to non-probiotic fermented soy yogurt, probiotic fermented soy yogurts are more acceptable in terms of mouth feel and visual appearance.

Conclusions

In our study, SUVBR enhanced the growth of *L. fermentum* BT 8219 cells to more than 9 log CFU mL^{-1} in biotin-soymilk after fermentation. The treatment also increased the intracellular and extracellular β-glucosidase activity of the lactobacilli, which in turn enhanced the bioconversion of isoflavone glucosides to aglycones in biotin-soymilk. This may have been due to the oxidative stress induced by SUVBR, which led to membrane permeabilisation and would have facilitated the exchange of molecules across the membrane. Such traits were only observed in the treated parent cells and were not passed on to the subsequent three subcultures of treated cells. SUVBR decreased the functional properties of the parent cells, such as tolerance to pH 2 and bile, antimicrobial activity and adherence ability. Acid tolerability

and adhesion ability of the treated cells improved in the subsequent subcultures of the treated cells while bile tolerability and antimicrobial properties persisted to first and second subcultures of the treated cells, respectively, possibly due to the longer recovery period needed for restoration of the UV-permeabilised membrane which involved energy and the synthesis of lipid. These results show that although the functional properties of *L. fermentum* were affected by the SUVBR, the treated cells still retained their functional properties. SUVBR could be utilised to produce putative probiotic cells with an increased β-glucosidase enzyme content to enhance the bioconversion of isoflavones glucosides to aglycones during fermentation.

Acknowledgements The authors would like to acknowledge the financial assistance provided by Universiti Sains Malaysia via the USM RU grant (1001/PTEKIND/815056), USM IPS Research Fund grant (1002/CIPS/ATTR 3100) and the Universiti Sains Malaysia Fellowship.

References

Azcarate-Peril MA, Tallon R, Klaenhammer TR (2009) Temporal gene expression and probiotic attributes of *Lactobacillus acidophilus* during growth in milk. J Dairy Sci 92:870–886

Baumstark-Khan C, Hentschel U, Nikandrova Y, Krug J, Horneck G (2000) Fluorometric analysis of DNA unwinding (FADU) as a method for detecting repair-induced DNA strand breaks in UV-irradiated mammalian cells. Photochem Photobiol 72:477–484

Berney M, Weilenmann HU, Egli T (2007) Adaptation to UVA radiation of *E. coli* growing in continuous culture. J Photochem Photobiol B Biol 86:149–159

Birošová L, Mikulášová M (2009) Development of triclosan and antibiotic resistance in *Salmonella enterica* serovar Typhimurium. J Med Microbiol 58:436–441

Blažeka B, Šuškovič J, Matošič S (1991) Antimicrobial activity of lactobacilli and streptococci. World J Microbiol Biotechnol 7:533–536

Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Cabiscol E, Tamarit J, Ros J (2000) Oxidative stress in bacteria and protein damage by reactive oxygen species. Int Microbiol 3:3–8

Chen RR (2007) Permeability issues in whole-cell bioprocess and cellular membrane engineering. Appl Microbiol Biotechnol 74:730–738

Cotter PD, Hill C (2003) Surviving the acid test: responses of gram-positive bacteria to low pH. Microbiol Mol Biol Rev 67:429–453

Cronan JE Jr, Waldrop GL (2002) Multi-subunit acetyl-CoA carboxylases. Prog Lipid Res 41:407–435

Davis MS, Solbiati J, Cronan JE Jr (2000) Overproduction of acetyl-CoA carboxylase activity increases the rate of fatty acid biosynthesis in *Escherichia coli*. J Biol Chem 275:28593–28598

Donkor ON, Henriksson A, Vasiljevic T, Shah NP (2007) Rheological properties and sensory characteristics of set-type soy yogurt. J Agric Food Chem 55:9868–9876

Elkins CA, Mullis LB (2004) Bile-mediated aminoglycoside sensitivity in *Lactobacillus* species likely results from increased membrane permeability attributable to cholic acid. Appl Environ Microbiol 70:7200–7209

Ewe JA, Wan-Abdullah WN, Liong MT (2010) Viability and growth characteristics of *Lactobacillus* in soymilk supplemented with B-vitamins. Int J Food Sci Nutr 61:87–107

Ewe JA, Wan-Abdullah WN, Liong MT (2011) ACE inhibitory activity and bioconversion of isoflavones by *Lactobacillus* in soymilk supplemented with B-vitamins. Br Food J 113:1127–1146

Ewe JA, Wan-Abdullah WN, Karim AA, Liong MT (2013) Ultraviolet radiation enhanced growth of *Lactobacillus* and their bioconversion of isoflavones in biotin-supplemented soymilk. LWT-Food Sci Technol 50:25–31

Forestier C, De Champs C, Vatoux C, Jolie B (2000) Probiotic activities of *Lactobacillus casei rhamnosus*: in vitro adherence to intestinal cells and antimicrobial properties. Res Microbiol 152:167–173

Gajic O, Buist G, Kojic M, Topisirovic L, Kuipers OP, Kok J (2003) Novel mechanism of bacteriocin secretion and immunity carried out by lactococcal MDR proteins. J Biol Chem 278:34291–34298

Golowczyc MA, Silva J, Teixeira P, De Antoni GL, Abraham AG (2011) Cellular injuries of spray-dried *Lactobacillus* spp. isolated from kefir and their impact on probiotic properties. Int J Food Microbiol 144:556–560

He YY, Klisch M, Häder DP (2002) Adaptation of cyanobacteria to UV-B stress correlated with oxidative stress and oxidative damage. Photochem Photobiol 76:188–196

Heppel LA (1969) The effect of osmotic shock on release of bacterial proteins and on active transport. J Gen Physiol 54:95–113

Herrling T, Fuchs J, Rehberg J, Groth N (2003) UV-induced free radicals in the skin detected by ESR spectroscopy and imaging using nitroxides. Free Radic Biol Med 35:59–67

Hong SI, Kim YJ, Pyun YR (1999) Acid tolerance of *Lactobacillus plantarum* from *Kimchi*. LWT-Food Sci Technol 32:142–148

Kano M, Takayanagi T, Harada K, Sawada S, Ishikawa F (2006) Bioavailability of isoflavones after ingestion of soy beverages in healthy adults. J Nutr 136:2291–2296

Kimoto H, Ohmomo S, Okamoto T (2002) Enhancement of bile tolerance in lactococci by Tween 80. J Appl Microbiol 92:41–46

Kirjavainen PV, Ouwehand AC, Isolauri E, Salminen SJ (1998) The ability of probiotic bacteria to bind to human intestinal mucus. FEMS Microbiol Lett 67:185–189

Krishnamurthy K, Tewari JC, Irudayaraj J, Demirci A (2010) Microscopic and spectroscopic evaluation of inactivation of *Staphylococcus aureus* by pulsed UV light and infrared heating. Food Bioprocess Technol 3:93–104

Larkin T, Price WE, Astheimer L (2008) The key importance of soy isoflavone bioavailability to understanding health benefits. Crit Rev Food Sci Nutr 48:538–552

Lin MY, Chang FJ (2000) Antioxidative effect of intestinal bacteria *Bifidobacterium longum* ATCC 15708 and *Lactobacillus acidophilus* ACTT 4356. Dig Dis Sci 45:1617–1622

Lin MY, Yen CL (1999) Antioxidative ability of lactic acid bacteria. J Agric Food Chem 47:1460–1466

Liong MT, Shah NP (2005) Production of organic acids from fermentation of mannitol, FOS and inulin by a cholesterol removing *Lactobacillus acidophilus* strain. J Appl Microbiol 99:783–793

Liong MT, Dunshea FR, Shah NP (2007) Effects of a synbiotic containing *Lactobacillus acidophilus* ATCC 4962 on plasma lipid profiles and morphology of erythrocytes in hypercholesterolaemic pigs on high- and low-fat diets. Br J Nutr 98:736–744

Liu P, Duan W, Wang Q, Li X (2010) The damage of outer membrane of *Escherichia coli* in the presence of TiO_2 combined with UV light. Colloids Surf B: Biointerfaces 78:171–176

Mahajan PM, Desai KM, Lele SS (2012) Production of cell membrane-bound α- and β-glucosidase by *Lactobacillus acidophilus*. Food Bioprocess Technol 5:706–718

Matallana-Surget S, Joux F, Wattiez R, Lebaron P (2012) Proteome analysis of UVB-resistant marine bacterium *Photobacterium angustum* S14. PLOSone 7:e42299

Murga MLF, Bernik D, de Valdez DF, Disalvo AE (1999) Permeability and stability properties of membranes formed by lipids extracted from *Lactobacillus acidophilus* grown at different temperatures. Arch Biochem Biophys 364:115–121

Nagira T, Narisawa J, Teruya K, Katakura Y, Shim SY, Kusumoto K, Tokumaru S, Tokumaru S, Barnes DW, Shirahata S (2002) Suppression of UVC-induced cell damage and enhancement of DNA repair by the fermented milk, Kefir. Cytotechnology 40: 125–137

Perluigi M, Di Domenico F, Blarzino C, Foppoli C, Cini C, Giorgi A, Grillo C, De Marco F, Butterfield DA, Schininà ME, Coccia R (2010) Effects of UVB-induced oxidative stress on protein expression and specific protein oxidation in normal human epithelial keratinocytes: a proteomic approach. Proteome Sci 8:1–14

Prasad J, McJarrow P, Gopal P (2003) Heat and osmotic stress responses of probiotic *Lactobacillus rhamnosus* HN001 (DR20) in relation to viability after drying. Appl Environ Microbiol 69:917–925

Ren MQ, Kuhn G, Wegner J, Chen J (2001) Isoflavones, substances with multi-biological and clinical properties. Eur J Nutr 40:135–146

Schär-Zammaretti P, Ubbink J (2003) The cell wall of lactic acid bacteria: surface and macromolecular conformations. Biophys J 85:4076–4092

Schillinger U, Guigas C, Holzapfel WH (2005) *In vitro* adherence and other properties of lactobacilli used in probiotic yogurt-like products. Int Dairy J 15:1289–1297

Setchell KDR, Brown NM, Zimmer-Nechemias L, Brasheas WT, Wolfe BE, Krischner AS, Heubi JE (2002) Evidence for the lack of absorption of soy isoflavone glycosides in humans, supporting the crucial role of intestinal metabolism for bioavailability. Am J Clin Nutr 76:447–453

Shiu CT, Lee TS (2005) Ultraviolet –B-induced oxidative stress and responses of the ascorbate-glutathione cycle in a marine macroalga *Ulva fasciata*. J Exp Bot 56:2851–2865

Smith HL, Howland MC, Szmodis AW, Li Q, Daemon LL, Parikh AN, Majewski J (2009) Early stages of oxidative stress-induced membrane permeabilization: a neuron reflectometry study. J Am Chem Soc 131:3631–3638

Šušković J, Kos B, Beganović J, Pavunc AL, Habjanič K, Matošić S (2010) Antimicrobial activity – the most important property of probiotic and starter lactic acid bacteria. Food Technol Biotechnol 48:296–307

Teh SS, Ahmad R, Wan Abdullah WN, Liong MT (2009) Evaluation of agrowastes as immobilizers for probiotics in soymilk. J Agric Food Chem 57:10187–10198

Ukuku DO, Geveke DJ (2010) A combined treatment of UV-light and radio frequency electric field for the inactivation of *Escherichia coli* K-12 in apple juice. Int J Food Microbiol 138:50–55

Vaughan EE, Mollet B, Devos WM (1999) Functionality of probiotics and intestinal lactobacilli: light in the intestinal tract tunnel. Curr Opin Biotechnol 10:505–510

Wei QK, Chen TR, Chen JT (2007) Using of *Lactobacillus* and *Bifidobacterium* to product the isoflavone aglycones in fermented soymilk. Int J Food Microbiol 117:120–124

Xu GQ, Chu J, Zhuang YP, Wang YH, Zhang SL (2008) Effects of vitamins on the lactic acid biosynthesis of *Lactobacillus paracasei* NERCB 0401. Biochem Eng J 38:189–197

Yokota A, Veenstra M, van Kurdi P, Veen HW, Konings WN (2000) Cholate resistance in *Lactococcus lactis* is mediated by an ATP-dependent multispecific organic anion transporter. J Bacteriol 182: 5196–5201