Journal of Functional Foods 64 (2020) 103571

Contents lists available at ScienceDirect

Journal of Functional Foods

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

Effects of supplementation of citrulline and *Lactobacillus helveticus* ASCC 511 on intestinal epithelial cell integrity

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ARTICLE INFO

Keywords:
Citrulline
Lactobacillus helveticus
Cell integrity
Tight junction
Anti-adhesion effect

ABSTRACT

Citrulline is a precursor of arginine and is believed to have the same beneficial effects as arginine. *Lactobacillus helveticus* ASCC 511 (LH511) utilizes arginine to produce extra energy for cell growth via arginine deiminase (ADI) pathway. Supplementation of both citrulline and LH511 is considered to be beneficial to intestinal tract. LH511 + Cit-2 mM was tested with IPEC-J2 cells to determine (i) the anti-adhesion effect against pathogenic infection (ii) effect on cell integrity by measuring transepithelial electrical resistance (TEER) and (iii) effect on tight junction (TJ)-proteins expression by qPCR and western-blot analyses. LH511 + Cit-2 mM exhibited a protective effect against adhesion of enterohemorrhagic (EHEC) and enteroinvasive (EIEC) *Escherichia coli*; it stimulated nitric oxide (NO) production, improved TEER and stimulated TJ-proteins expression. LH511 + Cit-2 mM showed better effects than that with arginine and citrulline alone. This study suggests supplementation of citrulline with LH511 has a synergistic effect and it might be a potential supplement for enhancing the health of the intestine.

1. Introduction

The intestinal mucosal barrier is the first line of defense in protecting the gastrointestinal tract from inflections by blocking the entry of pathogens and toxins. It is regulated by tight junction (TJ) proteins, components of the apical junctional complex found on the lateral membrane of the epithelial cells, through forming a paracellular space between adjacent epithelial cells (Turner, 2009). TJ consists of transmembrane proteins (including claudin proteins, occludin and junctional adhesion molecules (JAM)) that control the closing of the intercellular space and plaque proteins (including zonula occluden (ZO) family) that link with transmembrane proteins to perijunctional ring of actin and myosin (Ewaschuk et al., 2008; Mitic & Anderson, 1998; Ulluwishewa et al., 2011). TJ plays an important role in maintaining the intestinal permeability by controlling the transport of fluid through the intercellular space (Artis, 2008; Mitic & Anderson, 1998; Turner, 2009).

Intestinal mucosal barrier dysfunction is linked to different intestinal diseases, such as inflammatory bowel diseases (IBDs) (Anderson et al., 2010), infectious enteritis, irritable bowel syndrome (IBS) and diarrhea (Yang et al., 2015). Consequently, enhancing TJ functions is a useful strategy for maintaining intestinal health. Probiotics, arginine, and citrulline exert several beneficial effects on the intestinal barrier homeostasis, including the prevention of pathogenic translocation, regulation of intestinal permeability and stimulation of immune response (Andrade et al., 2015).

Probiotics are defined as live microorganisms; when administered in adequate amount, they contribute to health benefits on the intestinal tract of the host (FAO/WHO, 2001). Through regulation of the small intestinal permeability in atopic dermatitis patients (Rosenfeldt, Benfeldt, Valerius, Paerregaard, & Michaelsen, 2004), oral supplementation of probiotics has been reported to relieve the symptoms of several intestinal diseases (Ganji-Arjenaki & Rafieian-Kopaei, 2018; Shin et al., 2018). Numerous earlier studies reported that probiotics exerted protection and/or enhancement of the intestinal mucosal barrier function via modifying the TJ proteins both *in vitro* (Jariwala, Mandal, & Bagchi, 2017; Johnson-Henry, Donato, Shen-Tu, Gordanpour, & Sherman, 2008; Karczewski et al., 2010) and *in vivo* (Briskey et al., 2016; Mennigen et al., 2009; Patel et al., 2012; Ritze et al., 2014; Ukena et al., 2007). A recent animal study has demonstrated that ingestion of *Lactobacillus reuteri* R2LC modified ZO-1 and occludin expression in mice with dextran sulphate sodium-induced colitis (Ahl et al., 2016).

Arginine is an essential amino acid and a nitrogenous precursor of nitric oxide (NO) that is generated by NO synthase (NOS) (Boger & Bode-Boger, 2001). NO plays an important role in regulating various physiology in the cardiovascular, nervous, immune and gastrointestinal

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https://doi.org/10.1016/j.jff.2019.103571
Received 11 February 2019; Received in revised form 28 August 2019; Accepted 12 September 2019
Available online 31 October 2019

systems (Salzman et al., 1995). For gastrointestinal tract, NO has been found to modulate epithelial permeability (Kubes, 1992; Payne & Kubes, 1993), gastrointestinal motility (Burleigh, 1992), mucosal blood flow (Salzman et al., 1995) and vascular tone (Stark & Szurszewski, 1992). Arginine has been observed to regulate protein synthesis and intestinal restitution (Wu et al., 2007, 2009), and activate the protein expression level of ZO-1, occludin and claudin-1 in the intestinal epithelial cells (Beutheu, Ghouzali, Galas, Dechelotte, & Coeffier, 2013; Xia, Ye, Hou, & Yu, 2016). Citrulline is a non-protein amino acid and a precursor of arginine, catalysed by argininosuccinate synthetase (ASS); it first gets converted into argininosuccinate, which is then converted into arginine via argininosuccinate lyase (ASL) (Curis et al., 2005). Since citrulline is able to re-generate arginine, it is believed to act like arginine and promote the same functions as arginine (Bahri et al., 2013). In addition, intake of a high dosage of arginine causes adverse gastrointestinal effects, such as abdominal cramping and bloating, stomach cramping and diarrhea, but these symptoms are not reported for citrulline (Grimble, 2007). Some researchers have shown that citrulline is more effective than arginine in increasing arginine bioavailability during endotoxemia (Wijnands et al., 2012) and after massive intestinal resection (Osowska, Moinard, Neveux, Loi, & Cynober, 2004). Citrulline also exhibits similar protective effects as arginine against hypoxia-induced injury in intestinal epithelial cells (Chapman, Liu, Zhu, & Rhoads, 2012). Recently, Sellmann, Jin, Engstler, De Bandt, and Bergheim (2017) and Jegatheesan et al. (2016) reported the effects of citrulline on the intestinal barrier functions and showed that citrulline ameliorated ZO-1, occludin and claudin-1 expression in non-alcoholic liver disease (NAFLD) in mice.

Certain *Lactobacillus* strains are able to catabolize arginine and citrulline to produce ATP, ammonia, ornithine and carbamyl phosphate via arginine deiminase (ADI) pathway. A sufficient amount of active probiotic bacteria is necessary to contribute to health benefits (Sarao & Arora, 2017). The extra energy produced via ADI pathway is suggested to improve the probiotics growth and increase the probiotic concentration (Savino, Sanchez, Saguir, & De Nadra, 2012). *Lactobacillus helveticus* is a potential strain able to catabolize arginine/citrulline via ADI pathway (KEGG, 2017). Therefore, supplementation of citrulline with *L. helveticus* is potentially promoting the synergistic effect to intestinal barrier functions. This similar view has been reported by Adawi, Kasravi, Molin, and Jeppsson (1997) and Rishi, Bharrhan, Singh, and Kaur (2011), who determined the combination of *L. plantarum* and arginine exhibited the synergistic effect against liver damage *in vivo* model and the supplementation of arginine increased the concentration of *L. plantarum*. In addition, arginine has been suggested to be used as a prebiotic (Nascimento, 2018), which is defined as the fermented ingredient that alters the composition and/or activity of the gastrointestinal microflora, and provides advantageous effects on host well-being and health (Roberfroid, 2007).

In this study, we hypothesized that addition of citrulline along with *Lactobacillus* strains has the synergistic effect on the protective effect against pathogen adhesion on intestinal epithelial cells and the enhancing effect on the cell integrity and TJ functions. Moreover, there is no previous study comparing the effects of arginine and citrulline or investigating the effects of a combination of probiotics and citrulline on the intestinal barrier functions. Thus, the aims of this study were to investigate protective effect of supplementation of citrulline along with *Lactobacillus helveticus* ASCC 511 (LH511) on intestinal barrier functions and to compare the efficacy between arginine and citrulline treatments.

2. Materials and methods

2.1. Cell culture

The IPEC-J2 cell line was donated by Dr. Wai Hung Sit, from the Department of Biological Sciences, The University of Hong Kong. Cells were grown in Dulbecco's Modified Eagle Medium (DMEM)/F-12 (GIBCO®, Life Technologies; Spain), supplemented with 5% fetal bovine serum (Thermo Fisher Scientific Inc., Waltham, Massachusetts, United States), 1% ITS solution with insulin, transferrin, selenium and ethanolamine (GIBCO®, Life Technologies; Spain) and kept at 37 °C in a humidified 5% CO_2 atmosphere. IPEC-J2 cells were seeded in different types of tissue plates at 5×10^5 cell/ml cell concentration to conduct different assays (see below) when the cells reached 80% confluence.

2.2. Bacterial strains and preparation

Lactobacillus helveticus ASCC 511 (LH511) was obtained from the former Dairy Innovation Australia Limited (ASCC, Werribee, Victoria, Australia). It was stored at −80 °C in 20% (v/v) glycerol (Sigma-Aldrich, Munich, Germany) and activated in 10 ml of de Man, Rogosa and Sharpe (MRS) broth (BD, Franklin Lakes, New Jersey, USA) at 37 °C for 24 h. The activated cells were centrifuged at 5000*g* at 4 °C for 10 min and washed in phosphate buffered saline (PBS) and then re-suspended in PBS to obtain approximately 3×10^7 CFU/ml of bacteria.

2.3. MTT assay

To determine the effect of citrulline and LH511 on IPEC-J2 cell, the MTT assay was employed as per Stadelmann, Merino, Persson, and Svard (2012) with some modifications. IPEC-J2 cells were seeded in 96-well tissue culture plates (NUNC, Thermo Fisher Scientific, Waltham, Massachusetts, USA) until 80% confluence, and divided into groups as follows: control (without any treatment), Arg-2 mM (treated with 2 mM arginine), Cit-2 mM (treated with 2 mM citrulline), Cit-4 mM (treated with 4 mM citrulline), LH511 (treated with 3×10^7 CFU/ml LH511), LH511 + Cit-2 mM (treated with 2 mM citrulline and 3×10^7 CFU/ml LH511). The concentration of active cells was determined at 24 h interval × 3 until 72 h by MTT assay. MTT assay was performed by adding 10 µl of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) (5 mg/ml) to the well and incubated at 37 °C for 2 h. The medium was then discarded and 100 µl of dimethylsufoxide (DMSO) was added in well and incubated at 37 °C for 30 min. The absorbance was measured at 570 nm in an ELISA reader.

2.4. Antibacterial effect

2.4.1. Pathogenic strains

Enterohaemorrhagic *E. coli* PELI0480 (O157:H7) and enteroinvasive (EIEC) *E. coli* NFM138 (NFM138) were obtained from Prof. Dr. Hua Wei, State Key Laboratory of Food Science and Technology, Nanchang University and Prof. Dr. Wei Chen, State Key Laboratory of Food Science and Technology, Jiangnan University. All pathogens were cultivated in LB broth at 37 °C for 18–20 h before the assays.

2.4.2. Adhesion assay

IPEC-J2 cells were seeded in 12 well tissue plates until 80% confluence. Cells were washed two times with PBS and then 3×10^7 CFU/ml of *E. coli* O157:H7 and NFM138 and treatments were simultaneously added in the wells. The following treatments were used: control (without any treatment), Arg-2 mM (treated with 2 mM arginine), Cit-2 mM (treated with 2 mM citrulline), Cit-4 mM (treated with 4 mM citrulline), LH511 (treated with 3×10^7 CFU/ml LH511) and LH511 + Cit-2 mM (treated with 2 mM citrulline and 3×10^7 CFU/ml LH511). After 2 h incubation, the supernatant was discarded and the wells were washed with PBS and adhered cells were trypsinized by 0.25% of trypsin-EDTA solution (GIBCO®, Life Technologies; Madrid, Spain) and the viable bacteria attached to cell line were plated in the appropriate agar media. The number of CFU of LH511 and *E. coli* was evaluated using plate counting on MRS agar plates and LB agar plates, respectively. The percentage of adhesion was calculated by the following equation:

Relative Percentage of Adhesion (%) = ($CFU_{sample}/CFU_{control}$) × 100,

where CFU_{sample} was the number of bacteria adhered in the sample and $CFU_{control}$ was the number of bacteria adhered in the control.

2.5. Nitric oxide production measurement in IPEC-J2 cell line

Induction of NO production by the IPEC-J2 cells when exposed to the different treatments was assessed as per Pipenbaher et al. (2009) with some modifications.

IPEC-J2 cells were seeded in 96-well tissue culture plates until 80% confluence. The following treatments were used: control (without any treatment), Arg-2 mM (treated with 2 mM arginine), Cit-2 mM (treated with 2 mM citrulline), Cit-4 mM (treated with 4 mM citrulline), LH511 (treated with 3×10^7 CFU/ml LH511) and LH511 + Cit-2 mM group (treated with 2 mM citrulline and 3×10^7 CFU/ml LH511) and incubated for 24 h at 37 °C for 24 h in a humidified 5% CO_2 atmosphere. NO production induced by different treatment was measured by Griess reaction method (Green et al., 1982). One hundred microliter of cell culture mixture was transferred to a new 96-well plate and 100 µl Griess reagent (modified) (Sigma-Aldrich, Munich, Germany) was added into each of the wells and incubated for 15 min then NO detected at 540 nm by spectrophotometer. The results were expressed as µM of nitrite produced.

2.6. Transepithelial electrical resistance assay (TEER)

To determine the effect of citrulline and LH511 on intestinal epithelial cell integrity, IPEC-J2 cells were seeded in a permeable 12 mm Transwell with 0.4 µm pore polyester membrane inserts (Corning, New York, USA) until 80% confluence, and divided into groups as follows: control (without any treatment), Arg-2 mM (treated with 2 mM arginine), Cit-2 mM (treated with 2 mM citrulline), Cit-2 (treated with 4 mM citrulline), LH511 (treated with 3×10^7 CFU/ml LH511), LH511 + Cit-2 mM (treated with 2 mM citrulline and 3×10^7 CFU/ml LH511). TEER was measured using an ohmmeter (model EVOM, WPI Inc., Sarasota, Florida, USA) at 0, 2, 4, 8, 12 and 24 h.

2.7. Real-time PCR

Total RNA was isolated from the cells using TRIzol™ Reagent (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA) according to the manufacturer's protocol. RNA quality and quantity were determined by a NanoDrop Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA). Extracted RNA sample was reverse-transcribed to complementary DNA (cDNA) using a PrimeScriptTM RT Master Mix kit (Takara, Japan). Real-time PCR was conducted using an Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems®, Foster City, California, USA) with SYBR Green PCR Master Mix (Takara, Japan). The PCR system consisted of 5 µl of SYBR Green qPCR Mix, 1 µl of cDNA, 0.2 µmol of each primer, 0.2 µmol reference dye and 3.4 µl of distilled water in a final volume of 20 µl. The primers are shon in Table 1. β-actin was used as a housekeeping gene for the PCR reaction.

Table 1
Primers used for real-time PCR.

Genes	Primers	Sequences (5′-3′)	Size (bp)	T (°C)
β-actin	Forward	TGCGGGACATCAAGGAGAAG	216	60
	Reverse	AGTTGAAGGTGGTCTCGTGG		
Claudin-1	Forward	GCAGCAGCTTCTTGCTTCTC	664	58
	Reverse	CTGGCATTGACTGGGGTCAT		
Occludin	Forward	ATCAACAAAGGCAACTCT	157	50
	Reverse	GCAGCAGCCATGTACTCT		
ZO-1	Forward	GAGTTTGATAGTGGCGTT	298	50
	Reverse	GTGGGAGGATGCTGTTGT		

2.8. Western blot analysis

2.8.1. Sample preparation

The IPEC-J2 cells were seeded in 35 mm dish plates until 80% confluence and divided into groups as follows: control (without any treatment), Arg-2 mM (treated with 2 mM arginine), Cit-2 mM (treated with 2 mM citrulline), Cit-4 mM (treated with 4 mM citrulline), LH511 (treated with 3×10^7 CFU/ml LH511), LH511 + Cit-2 mM (treated with 2 mM citrulline and 3×10^7 CFU/ml LH511). Cells were collected after 2 and 24 h.

2.8.2. Western blotting

The effect of citrulline and LH511 on the expression of TJ proteins in the IPEC-J2 cells was assessed as per Yang et al. (2015) with some modifications.

IPEC-J2 cells were scraped into ice-cold radio-immunoprecipitation assay (RIPA) buffer (150 mM sodium chloride, 1% NP-40, 0.25% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl at pH 7.4 and 1 mM EDTA) and maintained constant agitation for 15 min at 4 °C. The lysed IPEC-J2 cells were centrifuged at 12,000*g* at 4 °C for 10 min and the supernatant was collected. The supernatant was used for western blot analysis. Protein contents were determined using the Bradford's method (Bradford, 1976). Samples with same protein concentrations were added in 6 × loading buffer and heated in 100 °C for 5 min. Samples were electrophoresed on 9% SDS-PAGE and then transferred to polyvinylidene difluoride (PVDF) membranes at 100 V for 90 min. The membranes were blocked in 5% skim milk for 1 h and then incubated with primary antibodies against zonula occluden-1 (ZO-1) (Bioss Inc., United States), claudin-1 (Cell Signaling Technology, Danvers, MA), occludin (Abcam, Cambridge, United Kingdom) and β-actin (Cell Signaling Technology, Danvers, MA, USA) overnight at 4 °C. After an overnight incubation, the membranes were washed by TBST and incubated with horseradish peroxidase conjugated secondary antibodies (Sigma-Aldrich, Munich, Germany) for 1 h at room temperature (25 °C). The membranes were washed by TBST and then analyzed by Chemi-Doc™ XRS + imaging system (Bio-Rad, Hercules, California, USA).

2.9. Determination of apoptosis by flow cytometry

The sample preparation and determination of the anti-apoptosis effect of citrulline and LH511 on IPEC-J2 cell by a flow cytometry were followed as previously described by Tang, Liu, Wang, Yu, and Fang (2018) with some modifications. IPEC-J2 cells were seeded in 35 mm dish plates until 80% confluence and divided into the following groups: control (without any treatment), Arg-2 mM (treated with 2 mM arginine), Cit-2 mM (treated with 2 mM citrulline), Cit-4 mM (treated with 4 mM citrulline), LH511 (treated with 3×10^7 CFU/ml LH511), LH511 + Cit-2 mM (treated with 2 mM citrulline and 3×10^7 CFU/ml LH511). After 24 h incubation, the supernatant was discarded and the wells were washed with PBS and cells were trypsinized by 0.25% of trypsin-EDTA solution (GIBCO®, Life Technologies; Spain). Approximately 1×10^6 cells were collected and centrifuged at 1000*g* for 5 min. Cells were washed twice with ice-cold PBS and re-suspended in 1 ml of Annexin V binding buffer and then 100 µl of the cell solution was transferred to a 5 ml tube and 1 µl of FITC Annexin V and 1 µl of PI were added. After 15 min incubation at room temperature in the dark, 400 µl of binding buffer was added and then apoptotic cells were determined using flow cytometer (BD, Franklin Lakes, New Jersey, USA).

2.10. Statistical analysis

All data were analyzed using GraphPad Prism 7 Software (GraphPad Software, city, USA). One-way analysis of variance (ANOVA) was used with Tukey's multiple comparisons test to determine the difference between all treatment groups on anti-adhesion, MTT assay and flow cytometry. Results are expressed as mean ± SD. Results of adhesion of

probiotic bacteria were determined by *t*-test. Two-way repeated measure ANOVA with Tukey's multiple comparisons test was used to analyze the results of TEER, qPCR and western blot analysis.

3. Results and discussion

3.1. Cell model and treatments

In this study, IPEC-J2 cell line was used as the *in vitro* model. This cell line is a non-transformed and non-cancerous intestinal cell line, isolated from the jejunum of a neonatal piglet and has been reported as a reliable *in vitro* model for the intestinal epithelium (Arce, Ramirez-Boo, Lucena, & Garrido, 2010; Brosnahan & Brown, 2012; Schierack et al., 2006). It has been applied for examining the barrier functions for nutrient transport (Xia et al., 2016) and against virus (Guo et al., 2016) and/or pathogenic infections (Koh et al., 2008). Moreover, IPEC-J2 cells represent an environment closer to human physiology than other rodent cells do (Brosnahan & Brown, 2012). In addition, the results from IPEC-J2 cells show high similarly with the other human cell lines (Koh et al., 2008), and act as a reliable reference to translate into *in vivo* situation (Zakrzewski et al., 2013).

The IPEC-J2 cells were treated with different treatments: 2 mM L-arginine (Sigma, St Louis, MO, USA), 2 mM L-citrulline (Sigma, St Louis, MO, USA), 4 mM L-citrulline, 3×10^7 CFU/ml of LH511 and 2 mM L-citrulline with 3×10^7 CFU/ml of LH511. A previous study by Tan et al. (2010) reported that 0.35 mM of arginine prevents pathogenic lipopolysaccharide induced death of intestinal cells. Another recent study by Varasteh, Braber, Kraneveld, Garssen, and Fink-Gremmels (2018) demonstrated that dosages of 0.4, 1 and 4 mM of arginine are not cytotoxic to Caco-2 cells and reported the protective effects of 4 mM arginine on intestinal integrity under heat stress conditions. Moreover, the level of arginine in the succus entericus of normal human subjects after ingestion of a protein meal ranged from 0.36 to 1.86 mM (Grimble, 2007). Two millimolar arginine is chosen as it is barely above the natural level with minimum adverse effects of high dosage of arginine. And we chose the 2 mM and 4 mM concentration of citrulline to test whether there is any effect difference between lower and higher dosage of citrulline. A prior study showed that 10^7 CFU/ml concentration of *Lactobacillus* strains promoted positive effects on intestinal cell integrity (Lepine et al., 2018). Also, based on the preliminary result of MTT, combining 2 mM citrulline with LH511 was the optimal dosage compared with combined with 4 mM citrulline (data not shown).

3.2. Effect of arginine, citrulline and L. helveticus ASCC 511 on cell viability of IPEC-J2 cell lines

The growth of IPEC-J2, when treated with arginine, citrulline, LH511 and combination of citrulline with LH511, was determined by MTT assay (Fig. 1). We found that all treatments were not cytotoxic to IPEC-J2 cells during 72 h incubation ($P < 0.001$). Cell growth of IPEC-J2 was slightly increased when incubated with arginine and citrulline with 12–28% stimulation after 48 and 72 h incubation, respectively. It has been reported that arginine is important for cell growth (Lenaerts et al., 2007). Moreover, both arginine and citrulline have been described as an important component to stimulate the Caco-2/TC7 cell growth under arginine-depleted medium (Stadelmann et al., 2012). Treatment with LH511 + Cit-2 mM was found to significantly enhance the cell growth compared with the control after 24, 48 and 72 h incubation with 27%, 56% and 38% increases, respectively (all $P < 0.05$). The cell growth promoting effect of lactic acid bacteria on the intestinal epithelium observed in the earlier studies; *Bifidobacterium infantis* 35624, and *Lactobacillus salivarius* subspecies *salivarius* UCC118 were shown to improve the cell concentration of HT-29 cells after 24 h (O'hara et al., 2006); *Lactobacillus rhamnosus* GG is able to produce p75 and p40 protein that promote cell proliferation and activate Akt-P13K signaling pathway in mouse colon epithelial (MCE) cells (Yan et al., 2007).

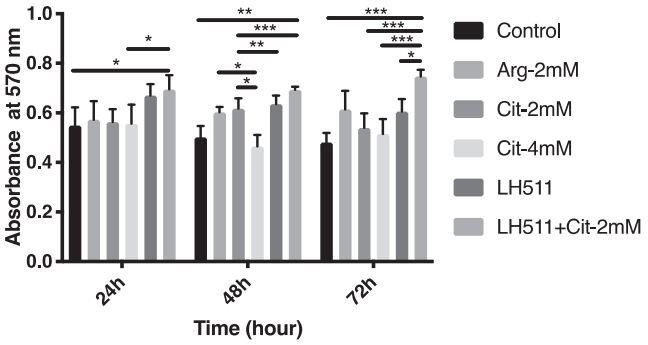

Fig. 1. Effects of arginine, citrulline and *L. helveticus* ASCC 511 on cell growth in IPEC-J2 cell at 24, 48 and 72 h by MTT assay. Arginine (2 mM) (Arg-2 mM), citrulline (2 mM and 4 mM) (Cit-2 mM and Cit-4 mM), *L. helveticus* ASCC 511 (LH511) (3×10^7 CFU/ml), and *L. helveticus* ASCC 511 with citrulline (LH511 + Cit-2 mM) (3×10^7 CFU/ml of *L. helveticus* ASCC 511 with 2 mM citrulline) were cultured in the IPEC-J2 cells (5×10^5 cell/ml) with MTT (5 mg/ml) and counted the number of live cells. Control without treatment. Results are represented as mean ± SEM, n = 5. Significance shown for the treatment as ***$P < 0.001$ by Two-way RM ANOVA and *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ as the difference compared with groups at the same time by Tukey's multiple comparisons test.

3.3. Anti-adhesion effect of arginine, citrulline and L. helveticus ASCC 511 against pathogenic E. coli and the effects of citrulline on adhesion of Lactobacillus helveticus ASCC 511 in IPEC-J2 cells

Adhesion to intestinal mucosal surface is the first step of pathogenic infection, thus inhibiting pathogenic adhesion is an effective way to protect from infection (Tuomola, Ouwehand, & Salminen, 1999). The protective effects of arginine, citrulline and LH511 against *E. coli* in the epithelial cells were examined against enterohemorrhagic *E. coli* (EHEC) and enteroinvasive *E. coli* (EIEC) and are shown in Fig. 2. All treatment groups significantly inhibited the adhesion of two *E. coli* strains in IPEC-J2 cells compared to the control ($P < 0.0001$). Our study found that 2 mM arginine reduced the adhesion of O157:H7 (EHEC) and NFM138 (EIEC) by 68% and 53%, respectively. The antibacterial ability of arginine was previously demonstrated in an *in vivo* study, which significantly enhanced the elimination of *Klebsiella pneumoniae*, methicillin resistant *Staphylococcus aureus* (MRSA), *Pseudomonas aeruginosa*, and human-derived and chicken-derived *E. coli* in several organs by stimulating the NO production to induce the phagocytosis (Chen et al., 2017). It is known that NO plays a major role on protection from bacterial infection (Nathan & Hibbs, 1991).

The inhibition effect of 2 mM and 4 mM citrulline against O157:H7 was 35% and 31% higher compared to 2 mM arginine, respectively, although it was not statistically significant ($P = 0.82$ and $P = 0.61$, respectively). Meanwhile, both dosage of citrulline showed a significant enhancement effect on inhibiting NFM138 (EIEC) adhesion compared to 2 mM arginine (both $P < 0.0001$). It is speculated that citrulline has a stronger inhibiting effect than arginine due to the ability to produce larger amount of NO by regenerating arginine by ASS and ASL. The mechanism of citrulline uptake in intestinal epithelium *in vitro* has been described previously (Vadgama & Evered, 1992). Extracellular citrulline can be transported across intestinal epithelial cells by different transport systems, mainly system $B^{0,+}$, L and $b^{0,+}$ (Bahri et al., 2008). The role of citrulline supplementation in this study is assumed to increase the endogenous citrulline level to enhance the arginine synthesis and eventually increase the epithelial NO production. To investigate whether the NO production can be stimulated by arginine, citrulline and LH511, we determined the concentration of NO in the cell culture supernatant by Griess reagent (Fig. 3). The NO concentrations in 4 mM citrulline, LH511 and LH511 + Cit-2 mM were significantly higher than

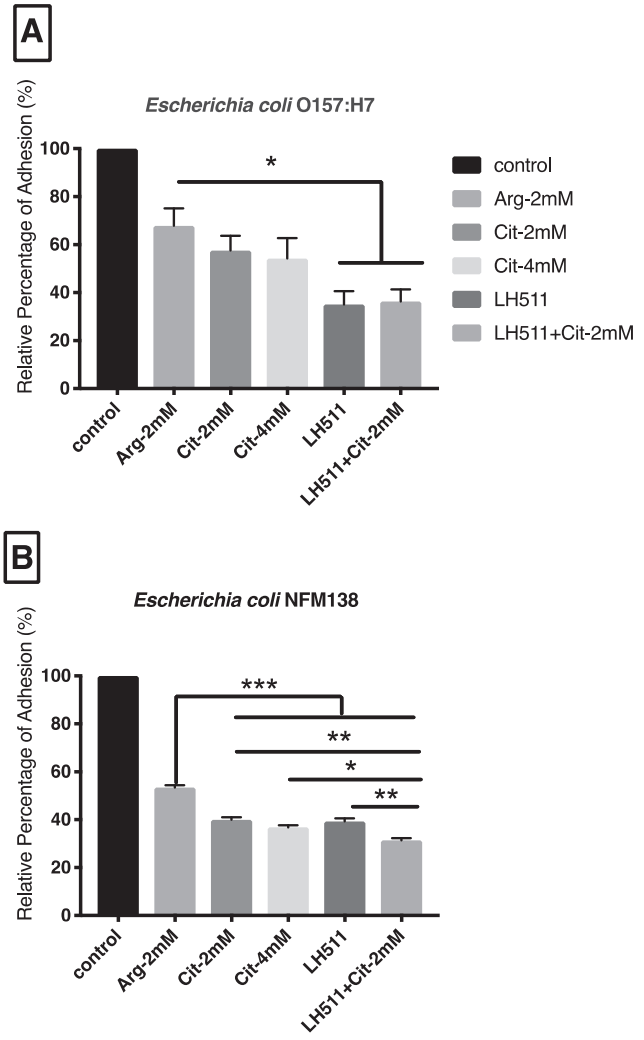

Fig. 2. Anti-adhesion effect of arginine, citrulline and *L. helveticus* ASCC 511 against (A) *Escherichia coli* PELI0480 (O157:H7) and (B) *Escherichia coli* NFM138 in IPEC-J2 cells. Arginine (2 mM) (Arg-2 mM), citrulline (2 mM and 4 mM) (Cit-2 mM and Cit-4 mM), and *L. helveticus* ASCC 511 with citrulline (LH511 + Cit-2 mM) (3×10^7 CFU/ml of *L. helveticus* ASCC 511 with 2 mM citrulline) was cultured in the IPEC-J2 cells (5×10^5 cell/ml) with *E. coli* strains (3×10^7 CFU/ml). The number of *E. coli* strains was counted and compared with control (no treatment, treated with *E. coli* alone). Results are represented as mean ± SEM, n = 3. Significance shown for the difference compared with control as ***P < 0.001 by One-way ANOVA and *P < 0.05, **P < 0.001 and ***P < 0.0001 as the difference compared between groups by Tukey's multiple comparisons test.

control (all P < 0.01). We found that NO concentration produced by 2 mM citrulline (11.95 ± 3.40 μM) was higher than 2 mM arginine (7.21 ± 3.73 μM) though it was not significantly different (P = 0.72). This difference might explain the higher anti-adhesion ability of 2 mM citrulline than 2 mM arginine. It is consistent with a recent *in vivo* study of Morita et al. (2014) that indicated citrulline generates higher level of NO than arginine, by comparing the change in plasma NO concentration after oral administration of same dosage of arginine and citrulline (both administrated 2.85 mmol/kg) in rats. In this regard, a prior *in vitro* study has persuasively demonstrated that citrulline supplementation stimulates NO production and improves the immune response against *Giardia intestinalis* infections in Caco-2 cells (Stadelmann, Hanevik, Andersson, Bruserud, & Svard, 2013). In addition, our result did not find any statistically significant dose-dependent effect in citrulline treatment on the anti-adhesion ability of both *E. coli* strains, even though the inhibition effect was slightly enhanced when the

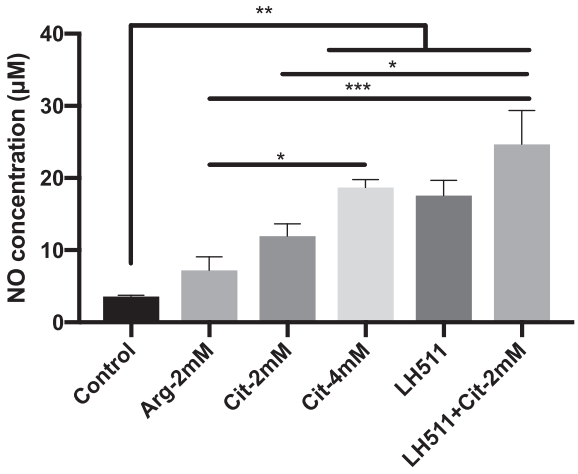

Fig. 3. Nitric oxide (NO) production stimulated by different treatments in IPEC-J2 cells. IPEC-J2 cells (5×10^5 cell/ml) were incubated with no treatment (control), Arginine (2 mM) (Arg-2 mM), citrulline (2 mM and 4 mM) (Cit-2 mM and Cit-4 mM), *L. helveticus* ASCC 511 (LH511) (3×10^7 CFU/ml) and *L. helveticus* ASCC 511 with citrulline (LH511 + Cit-2 mM) (3×10^7 CFU/ml of *L. helveticus* ASCC 511 with 2 mM citrulline) for 24 h. The NO concentrations were determined by Griess reaction method. Results are represented as mean ± SEM, n = 4. Significance shown for the difference compared with control as *P < 0.05 by One-way ANOVA and *P < 0.05, **P < 0.01 and ***P < 0.001 as the difference compared between groups by Tukey's multiple comparisons test.

concentration of citrulline was increased. This result might be related to NO production; we found that the NO concentration was increased from 11.95 ± 3.40 μM to 18.67 ± 2.23 μM when citrulline concentration increased from 2 mM to 4 mM although not significantly difference (P = 0.38). These findings suggest that citrulline promotes stronger anti-adhesion effect and NO production compared with the same dosage of arginine, and also the efficacy of citrulline might be enhanced by increasing the concentration.

LH511 has shown higher inhibition than 2 mM arginine against O157:H7 (EHEC) and NFM138 (EIEC) adhesion (both P < 0.05). The ability of probiotics on inhibiting pathogenic invasion has been described by other researchers (Wu et al., 2009). Resta-Lenert and Barrett (2003) showed that pre-treatment and simultaneous treatment with *Streptococcus thermophilus* and *Lactobacillus acidophilus* markedly reduced EIEC adhesion to Caco-2 cells. Another strain of LH, *L. helveticus* KS300 has been found to be able to inhibit uropathogenic *E. coli* IH111128 adhesion to HeLa cells (Atassi, Brassart, Grob, Graf, & Servin, 2006). The increased adhesion of probiotics to intestinal cells has been suggested to prevent the attachment of pathogens in cells. Fig. 4 shows the effect of citrulline on the adhesion of LH511 in IPEC-J2 cells. Interestingly, the adhesion of LH511 on epithelial cells was significantly enhanced by 9% when 2 mM citrulline was added (P < 0.05) and showed significantly higher anti-adhesion effect against NFM138 (EIEC) (P < 0.01). However, this did not strongly reduce the adhesion of O157:H7 compared with LH511. Tuomola et al. (1999) observed similar results, which suggested that strongly adhered *Lactobacillus rhamnosus* GG inhibited SfaII-fimbriated *E. coli* HB101 adhesion to human intestinal mucus but did not effectively inhibit against *Salmonella typhimurium* ATCC 14028 adhesions. These previous findings suggested that the adhesion-inhibiting effects of probiotics are not entirely related to their adhesion level but possibly dependent on the pathogenic strains. In the present study, the increased adherence of LH511 was more effective on inhibiting the adhesion of EIEC rather than EHEC. It might be due to the different mechanism on the interaction with the intestinal mucosa of EHEC and EIEC: EHEC first attaches to target cells surface, effaces microvillus and then delivers shiga toxin; whereas EIEC invades cell epithelium, lyses the endocytic

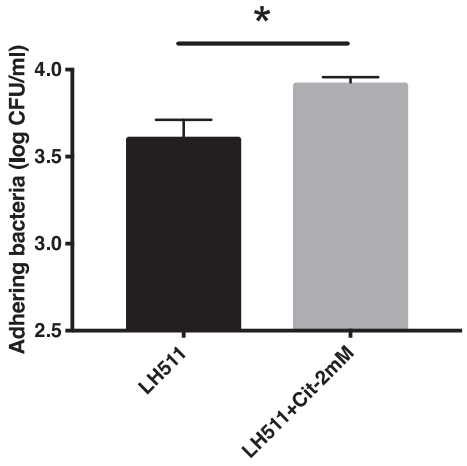

Fig. 4. Effect of 2 mM citrulline on adhesion of *L. helveticus* ASCC 511 in IPEC-J2 cells. *L. helveticus* ASCC 511 (LH511) (3×10^7 CFU/ml), and *L. helveticus* ASCC 511 with citrulline (LH511 + Cit-2 mM) (3×10^7 CFU/ml of *L. helveticus* ASCC 511 with 2 mM citrulline) was cultured in the IPEC-J2 cells (5×10^5 cell/ml) and counted the number of *L. helveticus* ASCC 511 growth. Results are represented as mean ± SEM, n = 3 and *P < 0.05 as determined by *t*-test.

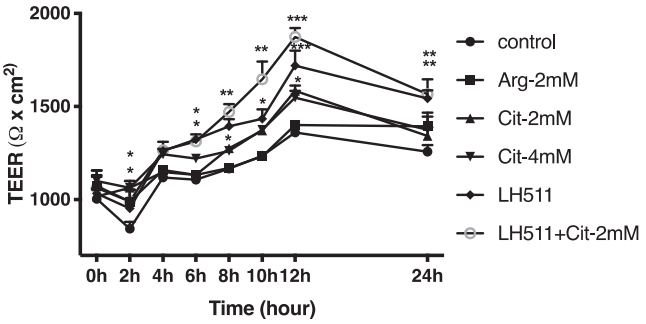

Fig. 5. Effects of arginine, citrulline and *L. helveticus* ASCC 511 on transepithelial electrical resistance (TEER) in IPEC-J2 cells over 24 h incubation. Arginine (2 mM) (Arg-2 mM), citrulline (2 mM and 4 mM) (Cit-2 mM and Cit-4 mM), *L. helveticus* ASCC 511 (LH511) (3×10^7 CFU/ml), and *L. helveticus* ASCC 511 with citrulline (LH511 + Cit-2 mM) (3×10^7 CFU/ml of *L. helveticus* ASCC 511 with 2 mM citrulline) were cultured in the IPEC-J2 cells (5×10^5 cell/ml) and TEER was measured at 0, 2, 4, 6, 8, 10, 12 and 24 h. Results are represented as mean ± SEM, n = 3. Significance shown for the time × treatment as **P < 0.01 by Two-way RM ANOVA and *P < 0.05, **P < 0.01 and ***P < 0.0001 as the difference compared with control at the same time point by Tukey's multiple comparisons test.

vacuole then laterally spread to adjacent epithelial cells (Nataro & Kaper, 1998). Therefore, our results indicate that strong-adhering LH511 might exert a stronger protective effect on the invasive pathogenic infection. Furthermore, we also found that NO concentration in IPEC-J2 cells was induced by LH511 and LH511 + Cit-2 mM at 17.57 ± 4.22 (P < 0.01) and 24.69 ± 9.34 μM (P < 0.001) compared with the control, respectively (Fig. 3). Some *Lactobacillus* strains have been found to capable of inducing NO production in the intestinal epithelium; however, the actual mechanism has not yet been confirmed (Pipenbaher et al., 2009). The ability of *Lactobacillus* strains to induce NO secretion in the intestinal epithelium was assumed to be either activating the bacterial or intestinal inducible nitric oxide synthase (iNOS) expression. A recent study from Yarullina et al. (2016) reported that NO formed from arginine by *L. plantarum* in the intestinal segment was caused by activating bacterial iNOS expression but not the intestinal iNOS. Thus, a further study is required to investigate the mechanism of NO formation of LH511 in the intestinal epithelium. Overall, our study suggests that LH511 + Cit-2 mM significantly improved the inhibition ability against NFM138 (EIEC) infection by reducing 14% and 9% adhesion to IPEC-J2 cells when compared to 2 mM citrulline and 4 mM citrulline, respectively. Thus, LH511 + Cit-2 mM has a greater effect to protect the intestinal epithelial cells against EIEC infection than other treatments in this study. Similar synergistic effect of supplementation of *Lactobacillus* strain with arginine has been reported to reduce the bacterial translocation and the amount of *Enterobacteriaceae* in the intestine in the rat model; the production of NO induced by arginine has been suggested as the cause of this effect (Adawi et al., 1997). In addition, the positive results of the anti-adhesion ability of LH511 + Cit-2 mM against EIEC and EHEC during *in vitro* infection suggest the potential capability to translate into *in vivo* model. This view is demonstrated by Wang et al. (2018), who examined the inhibitory ability of *L. reuteri* HCM2 against the growth of Enterotoxigenic *E. coli* (ETEC) and the adhesion level of ETEC to Caco-2 cells. They found the similar results to our current study: the sample probiotic bacteria have the inhibitory effects against diarrheic *E. coli* adhesion to intestinal epithelium. This prior study also demonstrated the effects of *L. reuteri* HCM2 *in vivo*, it showed that treatment of probiotics was effectively in reducing ETEC load in the jejunum, attenuating the damage of ETEC in intestinal morphology and modulating the gut microbiota in mice. Based on these previous findings, we postulate LH511 + Cit-2 mM might contribute beneficial effects on intestinal epithelium against diarrheic *E. coli* challenge when applied *in vivo* and further investigation is suggested.

3.4. Effects of arginine, citrulline and L. helveticus ASCC 511 in IPEC-J2 cell line viability, cell integrity and TJ protein expression

Several studies have demonstrated the important role of arginine in the gastrointestinal system by stimulating protein synthesis, maintaining intestinal mucosal barrier structure and functions and enhancing the intestinal mucosal regeneration after injury (Wu et al., 2009). Our findings suggest that supplementation with arginine, citrulline and LH511 contributes to positive effect on maintaining integrity and permeability of intestinal epithelial cells by measuring TEER (Fig. 5), which is used to evaluate the epithelial barrier function of paracellular ion permeability (Yang et al., 2015). Arginine is an essential component in the intestinal mucosal barrier and has been shown capable of restoring cell integrity after deprivation of arginine in cell medium (Xia et al., 2016). Chapman et al. (2012) found that both arginine and citrulline did not show significant improvement on TEER under normal conditions but markedly ameliorated the effects after hypoxia-induced injury. In contrast, our study found that citrulline was able to increase the TEER of IPEC-J2 cells compared with control at 2 h with either 2 mM and 4 mM concentration, as well as 2 mM citrulline at 12 h. Arginine, however, did not show significant improvement effect on TEER throughout 24 h incubation at 2 mM concentration level. The advantageous effects of probiotics on the intestinal epithelial barrier function have been suggested by previous studies (Ohland & Macnaughton, 2010). For example, *Lactobacillus reuteri* i5007, *Lactobacillus plantarum* MB452, *Streptococcus thermophilus* ATCC 19258 and *Lactobacillus acidophilus* ATCC 4356, respectively, stimulated the TEER of IPEC-J2 (Yang et al., 2015), Caco-2 (Anderson et al., 2010) and HT29/cl.19A cells (Resta-Lenert & Barrett, 2003). In recent years, other strains of *L. helveticus* have been reported to restore TEER on HT-29 and Caco-2 cells after Enteropathogenic *E. coli* (EPEC) O26:H11-induced injury (Jariwala et al., 2017). We found that LH511 and LH511 + Cit-2 mM significantly improved TEER from 6 to 24 h compared to the control and 2 mM arginine (all P < 0.05) and showed a time-dependent effect (P < 0.0001). Overall, LH511 + Cit-2 mM showed higher TEER enhancement than LH511 throughout 24 h incubation. However, LH511 + Cit-2 mM exhibited only a statistically significant higher effect than LH511 alone at 10 h (P < 0.05).

The TEER level is positively associated with the expression of occludin and ZO-1 protein. Anderson et al. (2010) showed that the TEER

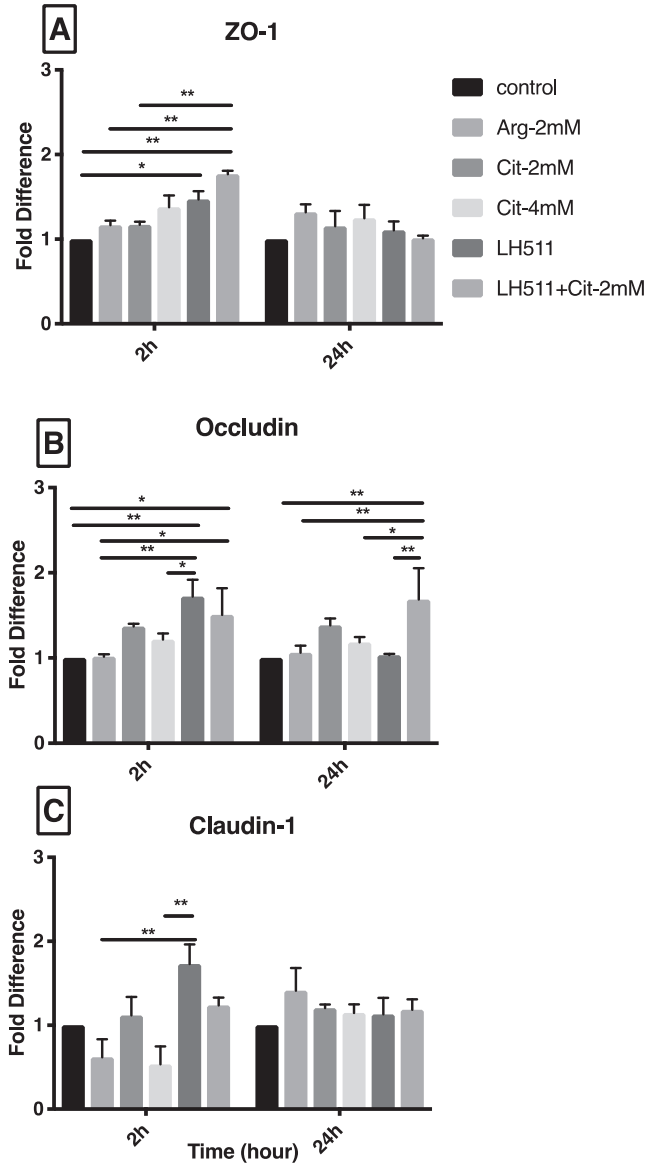

Fig. 6. The mRNA expression of tight junction proteins in IPEC-J2 cells under different treatments after 2 h and 24 h. IPEC-J2 cells (5×10^5 cell/ml) were incubated with no treatment (control), 2 mM arginine (Arg-2 mM), 2 mM or 4 mM citrulline (Cit-2 mM or Cit-4 mM), *L. helveticus* ASCC 511 (LH511) (3×10^7 CFU/ml), and *L. helveticus* ASCC 511 with citrulline (LH511 + Cit-2 mM) (3×10^7 CFU/ml of *L. helveticus* ASCC 511 with 2 mM citrulline) for 2 h and 24 h. The mRNA expression of ZO-1, occludin and claudin-1 was analyzed by real time PCR. Results are represented as mean ± SEM, n = 3. Significance shown for the time × treatment as *P < 0.05 and **P < 0.01 by Two-way RM ANOVA and *P < 0.05 and **P < 0.01 as the difference compared with groups at the same time by Tukey's multiple comparisons test.

and ZO-1 and occludin gene expression of Caco-2 cells was significantly modified after incubated with *L. plantarum* MB452 for 10 h. This view is also supported by our findings. We examined the effect of arginine, citrulline and LH511 on TJ (ZO-1, claudin-1 and occludin) protein expression in IPEC-J2 cells after 2 h and 24 h treatment using qPCR and western blot analysis (Figs. 6 and 7). We also found that the mRNA expression of occludin and ZO-1 in LH511 and LH511 + Cit-2 mM was significantly stimulated at 2 h and protein level of occludin and ZO-1 was stimulated at 24 h compared with control. It could explain that the TEER of LH511 and LH511 + Cit-2 mM group was not significantly affected at 2 h but then enhanced at 24 h. The mRNA expression of ZO-1 under LH511 and LH511 + Cit-2 mM treatment was up-regulated at 2 h and then returned at the normal level at 24 h, whereas the mRNA expression of occludin was up-regulated at 2 h, but it was continuously expressed at a higher level in LH511 + Cit-2 mM group at 24 h.

Meanwhile, the ZO-1 and occludin protein levels were not affected at 2 h, but it was significantly increased at 24 h compared to control (all P < 0.05). It might imply that ZO-1 and occludin protein syntheses were stimulated after 2 h treatment of LH511 and LH511 + Cit-2 mM. Protein transcription might be declined at 24 h by negative feedback mechanism, thus protein levels were increased but mRNA expression was returned to normal level at 24 h. Interestingly, the mRNA expression of occludin at 24 h after LH511 + Cit-2 mM treatment remained at a high level. Moreover, generally, LH511 and LH511 + Cit-2 mM had greater effects on both ZO-1 or occludin gene expression and protein synthesis when compared to 2 mM arginine and 2 mM citrulline. However, LH511 showed a stronger effect than LH511 + Cit-2 mM on claudin-1 mRNA and protein expression. Beneficial effects of modulating TJ protein by probiotics have been examined previously; Ewaschuk et al. (2008) have shown the expression of ZO-1, occludin and claudin-1, 3 and 4 in T84 cells was enhanced after 24 h incubated with *Bifidobacterium infantis*. *L. reuteri* i5007 has been reported bringing protective effects on *E. coli* Lipopolysaccharides (LPS)-treated IPEC-J2 cells with improved the mRNA expression and protein level of ZO-1, occludin and claudin-1 but not significant on non-LPS infected cells (Yang et al., 2015). Another study by Zhang et al. (2015) found that *L. rhamnosus* ATCC 7469 stimulated occludin expression in non-infected IPEC-J2 cells after 3 h and enhanced occludin and ZO-1 expression under enterotoxigenic *E. coli* (ETEC) infection. These previous findings suggest that probiotics improved intestinal barrier function through up-regulating the TJ proteins in non-infected cells and in response to pathogenic infection induced damage. Overall, our data suggested that the treatment with LH511 + Cit-2 mM has the enhancing effects on cell integrity and TJ proteins in normal IPEC-J2 cells. In addition, the possibility of positive *in vitro* results to translate to *in vivo* study was examined by the earlier study of Yang et al. (2015). It indicated *L. reuteri* i5007 at 3×10^7 CFU/ml enhanced TEER value and TJ proteins expression in IPEC-J2 cells; it also demonstrated a 14-days oral treatment of 6×10^9 CFU/ml of *L. reuteri* i5007 have the same effect on TJ proteins (ZO-1, occludin and claudin-1) expression on jejunum and ileum in newborn piglets. It provided evidence that positive effects of LH511 + Cit-2 mM displayed *in vitro* model in this study speculate similar effects *in vivo* model to up-regulate TJ proteins expression to improve the intestinal mucosal barrier function. Moreover, it is noteworthy to mention that LH511 + Cit-2 mM were able to improve the expression of TJ proteins under normal condition even without pathogenic infections in our current study. Given the positive effects of LH511 + Cit-2 mM, it may be considered a potential novel dietary supplement to enhance the intestinal barrier functions.

3.5. Effects of arginine, citrulline and L. helveticus ASCC 511 in IPEC-J2 cells apoptosis

Apoptosis plays an important role in the homeostatic balance of the intestinal epithelium (Ramachandran, Madesh, & Balasubramanian, 2000; Wang et al., 2008). Effects of arginine, citrulline, and LH511 on apoptosis of IPEC-J2 cells are shown in Fig. 8. The apoptosis decreased in IPEC-J2 cells incubated with 2 mM arginine, 2 mM and 4 mM citrulline when compared to the control (both P < 0.05). The apoptosis-inhibiting effect of arginine and citrulline exhibited in our study could be explained by the NO production ability. NO prevents apoptosis via increasing Bcl-2 expression and down-regulating caspase-3 activity by S-nitrosylation of the caspase catalytic site of cysteine (Ramachandran et al., 2000). LH511 co-incubated with 2 mM citrulline also resulted in the reduction of apoptosis in IPEC-J2 cells (P < 0.01). Another *Lactobacillus* strains, *L. rhamnosus* ATCC 7469, is also found to reduce late apoptosis in normal and ETEC infected IPEC-J2 cells (Zhang et al., 2015). The ability to inhibit apoptosis on protecting normal cells is

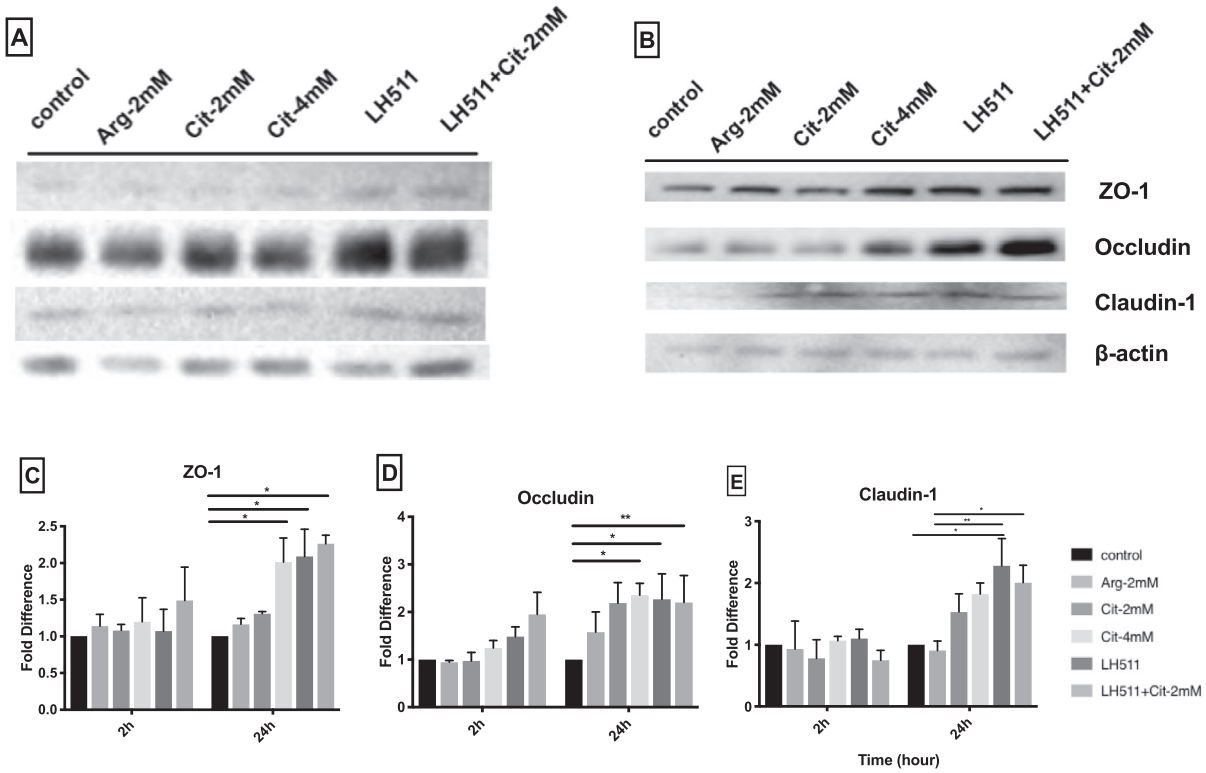

Fig. 7. Effects of different treatments on the tight junction proteins expression levels in IPEC-J2 cells after (A) 2 h and (B) 24 h. IPEC-J2 cells (5×10^5 cell/ml) were incubated with no treatment (control), 2 mM arginine (Arg-2 mM), 2 mM or 4 mM citrulline (Cit-2 mM or Cit-4 mM), *L. helveticus* ASCC 511 (LH511) (3×10^7 CFU/ml), and *L. helveticus* ASCC 511 with citrulline (LH511 + Cit-2 mM) (3×10^7 CFU/ml of *L. helveticus* ASCC 511 with 2 mM citrulline) for 2 h and 24 h and were analyzed by western blot analysis. The ratio of the (C) ZO-1 band intensity, (D) Occludin band intensity and (E) Claudin-1 band intensity to the beta actin band intensity. Results are represented as mean ± SEM, n = 3. Significance shown for the time × treatment as *P < 0.05 by Two-way RM ANOVA and *P < 0.05 and **P < 0.01 as the difference compared with groups at the same time by Tukey's multiple comparisons test.

important in clinical utility, especially when applied against toxic condition-therapy, similar to melatonin that shows protecting effect on normal cells by reducing apoptosis under the anticancer treatments but induced apoptosis of tumor cells (Sainz et al., 2003). Kahouli, Malhotra, Alaoui-Jamali, and Prakash (2015) and El-Deeb, Yassin, Al-Madboly, and El-Hawiet (2018) reported the beneficial effects of *Lactobacillus* strains against cancer, where *L. acidophilus* and *L. fermentum* stimulated apoptosis in colon cancer cells and increased the proliferation of normal cells. Anti-apoptotic effect of the treatment of LH511 + Cit-2 mM on normal cells in this study might be a potential therapy for protecting normal intestinal epithelial cells under harmful conditions. Thus, further study on this assumption of LH511 + Cit-2 mM is required.

4. Conclusions

In this study, we determined the effects of combinations of LH511 and citrulline on intestinal barrier integrity. Although our results indicated lower (2 mM) and higher (4 mM) concentration of citrulline did not show any significant dose-dependent effects, it showed an upward trend in TJ proteins expression when the dosage of citrulline is increased to 4 mM. This dose-response effect of citrulline was also reported by a prior study of Ham et al. (2015), which indicated that while 1 mM and 2.5 mM citrulline on murine C2C12 myoblasts showed the similar results, 5 mM citrulline promoted the retrograde result. Therefore, it is suggested both 2 mM and 4 mM concentration are an optimal and effective dosage to promote the positive effects on intestinal epithelium. We found that supplementation of arginine and citrulline stimulated NO concentration in IPEC-J2 cells. It may support the view

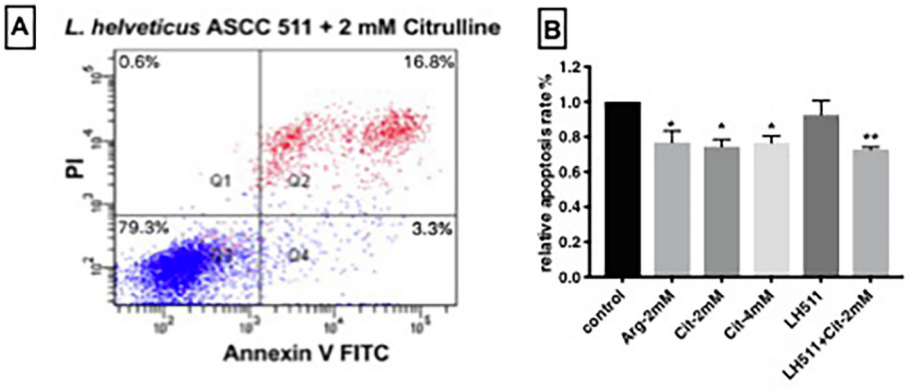

Fig. 8. Effect of arginine, citrulline and *L. helveticus* ASCC 511 on apoptosis in IPEC-J2 cells. (A) Representative flow cytometry dot plot of Annexin V FITC versus propidiun iodide and (B) the analysis of apoptosis change. IPEC-J2 cells (5×10^5 cell/ml) were incubated with no treatment (control), 2 mM arginine (Arg-2 mM), 2 mM or 4 mM citrulline (Cit-2 mM or Cit-4 mM), *L. helveticus* ASCC 511 (LH511) (3×10^7 CFU/ml), and *L. helveticus* ASCC 511 with citrulline (LH511 + Cit-2 mM) (3×10^7 CFU/ml of *L. helveticus* ASCC 511 with 2 mM citrulline) for 24 h and were analyzed by flow cytometric method. Results are represented as mean ± SEM, n = 3. Significance shown for the difference compared with control as *P < 0.05 and **P < 0.01 by One-way ANOVA.

that supplementation of arginine and citrulline increases the uptake across intestinal epithelium to enhance the NO production via increasing the endogenous levels arginine or citrulline. Indeed, understanding the metabolism of citrulline in intestinal epithelium should be further studied. Supplementation of 2 mM citrulline with LH511 is found superior to 4 mM citrulline as the significant stronger effects in anti-adhesion ability, TEER and TJ proteins expression. The addition of 2 mM citrulline with LH511 has also been found to strengthen the adhesion to intestinal epithelium as well as stimulate NO production. It is noteworthy as it might be useful for improving the intestinal immune system. These findings showed stronger effects than administration of arginine and citrulline alone. It is showed that citrulline has prebiotic-like ability, therefore, supplementation of citrulline with LH511 has the synergistic effect on improving intestinal epithelial barrier functions. It is possible that this combination could be a novel food supplementation strategy for improving intestinal health via enhancing the TJ functions.

Author contributions

The author's responsibilities were as follows: S.W.H designed and conducted the study and wrote the manuscript. H.E-N assisted in the western blot and TEER analysis. N.P.S. assisted in designing the study, revised the manuscript and edited the manuscript.

6. Ethics statement

This research did not include any human subjects and animal experiments.

Acknowledgments

The research was supported by grants from the University of Hong Kong. The authors are grateful to Dr. Wai Hung Sit and Mr. Ken Wong for their expert assistance with the cell cultures.

Declaration of Competing Interest

The authors declare no conflict of interest.

Appendix A

ADI	Arginine deminase
ANOVA	Analysis of variance
ASL	Argininosccinate lyase
ASS	Argininosuccinate synthetase
cDNA	complementary DNA
CK	Carbamate kinase
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsufoxide
EHEC	Enterohemorrhagic *Escherichia coli*
EIEC	Enteroinvasive *Escherichia coli*
EPEC	Enteropathogenic *Escherichia coli*
ETEC	Enterotoxigenic *Escherichia coli*
JAM	Junctional adhesion molecules
LH511	*Lactobacillus helveticus* ASCC 511
LPS	Lipopolysaccharides
IBD	Inflammatory Bowel Diseases
IBS	Irritable bowel syndrome
iNOS	Intestinal inducible nitric oxide synthase
MCE	Mouse colon epithelial
MRSA	Methicillin resistant *Staphylococcus aureus*
MRS	de Man, Rogosa and Sharpe
MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
NAFLD	Non alcoholic liver disease
NO	Nitric oxide
NOS	Nitric oxide synthase
OTC	Ornithine transcarbamylase
PVDF	Polyvinylidene difluoride
RIPA	Radio-immunoprecipitation assay
TEER	Transepithelial electrical resistance
TJ	Tight junction
ZO-1	Zonula occluden-1

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