

Full Paper

Isolation, identification, and impact on intestinal barrier integrity of *Lactiplantibacillus plantarum* from fresh tea leaves (*Camellia sinensis*)

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Lactic acid bacteria (LAB) are safe microorganisms that have been used in the processing of fermented food for centuries. The aim of this study was to isolate *Lactobacillus* from fresh tea leaves and examine the impact of an isolated strain on intestinal barrier integrity. First, the presence of *Lactobacillus* strains was investigated in fresh tea leaves from Kagoshima, Japan. Strains were isolated by growing on De Man, Rogosa and Sharpe (MRS) agar medium containing sodium carbonate, followed by the identification of one strain by polymerase chain reaction (PCR) and *pheS* sequence analysis, with the strain identified as *Lactiplantibacillus plantarum* and named *L. plantarum* LOC1. Second, the impact of strain LOC1 in its heat-inactivated form on intestinal barrier integrity was investigated. Strain LOC1, but not *L. plantarum* ATCC 14917^T or *L. plantarum* ATCC 8014, significantly suppressed dextran sulfate sodium (DSS)-induced decreases in transepithelial electrical resistance values of Caco-2:HT29-MTX 100:0 and 90:10 co-cultures. Moreover, in Caco-2:HT29-MTX co-cultures (90:10 and 75:25), levels of occludin mRNA were significantly increased by strain LOC1 compared with untreated co-cultures, and strain LOC1 had higher mRNA levels of MUC2 and MUC4 mucins than *L. plantarum* ATCC 14917^T and *L. plantarum* YT9. These results indicate that *L. plantarum* LOC1 may be used as a safe probiotic with beneficial effects on the intestinal barrier, suggesting that fresh tea leaves could be utilized as a safe source for isolating probiotics.

Key words: *Lactiplantibacillus plantarum*, fresh tea leaves, Caco-2/HT29-MTX cultures, barrier integrity, mucins

INTRODUCTION

Lactic acid bacteria (LAB) are commonly found in a diverse range of environments, from dairy products and meat to animals, sewage, and plants [1, 2]. Of the many genera collectively comprising LAB, *Lactobacillus* is one of the most important genera [3].

Many species comprise the genus *Lactobacillus* and taxonomically related genera, all of which bear the characteristics of gram-positive, non-spore-forming, catalase-negative rods. Following carbohydrate fermentation by these bacteria, lactic acid is produced as the major end product [2]. They are considered to be generally recognized as safe organisms and can be safely used as probiotics for medical and veterinary applications [4]. Probiotics, as defined in a Food and Agriculture Organization/World Health Organization (2002) report, are “live microorganisms which when administered in adequate amounts confer a health benefit on the host” [5].

Tea (*Camellia sinensis*, Theaceae) is consumed globally, with

only water being more widely imbibed. Drinking of tea has been associated with health benefits such as the lowering of cholesterol and protective effects against cardiovascular disease and cancer [6]. *Lactobacillus* strains and taxonomically related bacteria have been frequently isolated from fermented tea leaves. For example, *Limosilactobacillus fermentum* has been isolated from Thai traditional tea [7]. In addition, the isolation of six strains of *Lactocaseibacillus pantheris*, five strains of *Lactiplantibacillus pentosus*, and four strains of *Paucilactobacillus suebicus* from fermented tea leaves known as Miang has been reported [8]. Furthermore, *Lactiplantibacillus plantarum* has been found in Ishizuchi-kurocha and Awa-bancha [9]. Meanwhile, few studies have examined the isolation of *Lactobacillus* from fresh tea leaves. One study has reported the isolation of a strain of *Lactiplantibacillus paraplantarum* from fresh tea leaves (*C. sinensis*), which suggests that many important probiotic microorganisms may be found in tea leaves, which may thus represent a safe source of probiotics [10].

It has been reported that in an *in vitro* model of necrotizing

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enterocolitis, certain *Lactobacillus* strains have the ability to strengthen and maintain the intestinal barrier [11]. The expression levels of genes related to tight junction (TJ) formation [12] and barrier integrity of intestinal epithelial cell (IEC) monolayers, as measured by transepithelial electrical resistance (TEER), were increased [13–15]. In addition, probiotics were found to be able to actively secrete soluble mediators [16], promote TJ formation [17], induce mucin gene expression, and alter the composition of the mucus layer that can occur as a direct response to bacterial attachment to the epithelium [18], thereby protecting the intestinal barrier. Adherence of probiotics has been confirmed to be unique to both species and strains following studies using IECs such as monocultures of Caco-2 [13], monocultures of HT29 and its subclones [19], and co-cultures of Caco-2:HT29-MTX cells [20, 21].

The aim of this study was to isolate *Lactobacillus* strains from fresh tea leaves, identify a single strain by polymerase chain reaction (PCR) and sequencing of phenylalanyl-tRNA synthase alpha subunit (*pheS*), and then examine the impact of the selected strain on intestinal barrier integrity in a co-culture model of the small and large intestine. Mucin is primarily secreted by HT29-MTX cells, so co-culture models were established that combine HT29-MTX and Caco-2 cells at ratios that mimic the permeability characteristics of the human intestinal barrier in the small (90:10, Caco-2:HT29-MTX) and large intestines (75:25, Caco-2:HT29-MTX) [22]. Using TEER and PCR, we investigated the epithelial barrier integrity and expression of genes encoding TJs and mucin in Caco-2/HT29-MTX mono- or co-cultures to identify the enhancing effects of the isolated bacterial strain compared with a type strain. To the best of our knowledge, this is the first report of intestinal cell co-culture models being used to assess a *Lactobacillus* strain isolated from fresh tea leaves.

MATERIALS AND METHODS

Isolation of a *Lactobacillus* strain from fresh tea leaves

Fresh tea leaves from a Kagoshima tea plantation in Japan were used for bacterial isolation. They were added to 10 mL of phosphate buffered saline (PBS) and ground in a mortar with a pestle at room temperature. Fifty microliters of the solution were spread on De Man, Rogosa and Sharpe (MRS) agar (Oxoid, Basingstoke, UK) supplemented with sodium carbonate (Wako Pure Chemical Corporation, Osaka, Japan) at a final concentration of 1% and incubated at 37°C for 48 hr under anaerobic conditions using the AnaeroPack-Anaero system (Mitsubishi Gas Chemical Co., Inc., Tokyo, Japan). One of the isolates, designated strain LOC1, was randomly selected and inoculated into 10 mL MRS broth in a test tube with a screw cap. Strain LOC1 was cultured at 37°C for 1–2 days. Stock cultures were stored at –80°C in MRS broth containing 30% glycerol.

Identification of strain LOC1

Strain LOC1 was identified to the genus and species levels by PCR. Whole-genome DNA of strain LOC1 was prepared following the procedure of Marmur [23]. The primers used in this study are shown in Table 1 [24, 25]. The PCR mixture contained 250 ng of genomic DNA as the template, 100 pmol of each primer (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA), 5 U of Ex Taq DNA polymerase (Takara Bio, Shiga, Japan), 12.5 mM MgCl₂, and 200 mM each dNTP in deionized water to a final volume of 50 µL. *L. plantarum* ATCC 14917^T and *L. plantarum* ATCC 8014 were used as positive controls for the *Lactobacillus* PCR amplifications, and *Bifidobacterium lactis* Bb12 and distilled water were used as negative controls. *L. plantarum* ATCC 14917^T and *L. plantarum* ATCC 8014 were used as positive controls, and *Lactobacillus acidophilus* La5 and distilled water were used as negative controls during PCR amplification of *L. plantarum*.

The PCR conditions for the genus *Lactobacillus* and the species *L. plantarum* were described by Rinttilä *et al.* [24] and Matsuda *et al.* [25], respectively. For the genus *Lactobacillus*, the amplification program consisted of one cycle of 95°C for 5 min; 30 cycles of 95°C for 15 sec, 58°C for 20 sec, and 72°C for 45 sec; and finally one cycle of 72°C for 5 min using a SimpliAmp thermal cycler (Applied Biosystems, Foster City, CA, USA). For *L. plantarum*, the program comprised an initial denaturation at 95°C for 15 min; 40 cycles of 94°C for 20 sec, 60°C for 20 sec, and 72°C for 50 sec; and then one cycle of 72°C for 5 min. The PCR products were analyzed on 1% agarose gels, which were then stained with 0.5 gL⁻¹ (w/v) ethidium bromide and scanned by a FluoroPhoreStar 3000 (Anatech, Tokyo, Japan). Product sizes were identified using a 100 bp DNA ladder (Takara Bio) as a reference standard.

The use of *pheS* gene sequence analysis has been applied for species identification of the genus *Lactobacillus* [26, 27], so the identification of strain LOC1 was further verified by the *pheS* gene sequence using whole genome sequencing. Whole-genome DNA of strain LOC1 was prepared as described above. High-quality DNA was subjected to library preparation using a Nextera Flex kit (Illumina, San Diego, CA, USA) according to the manufacturer's recommendations, followed by paired-end sequencing using an Illumina iSeq 100. Raw WGS data (FASTQ files) underwent quality control (QC), filtering, trimming, and de novo assembly using Shovill (SPAdes, v3.12; using the –trim and –opts ‘–sc’ parameters; <https://github.com/tseemann/shovill>). Assemblies were annotated with Prokka 1.14.0 [28].

IEC co-culture conditions

The human colorectal adenocarcinoma cell line Caco-2 and the HT29-MTX cells were obtained from the American Type Culture Collection (Manassas, VA, USA). Caco-2 and HT29-MTX cells were used in experiments from passage 28–33 and 18–25, respectively.

Table 1. Primers used for this study

Name	Primer	Sequence	Amplicon Length (bp)	Refs
<i>Lactobacillus</i>	Forward	5-AGCAGTAGGGAATCTTCCA-3	341	[24]
	Reverse	5-CACCGCTACACATGGAG-3		
<i>L. plantarum</i>	Forward	5-CTCTGGTATTGATTGGTGCTTGCAT-3	54	[25]
	Reverse	5-GTTCGCCACTCACTCAAATGTA-3		

Caco-2 and HT29-MTX cells were separately cultured at a density of 0.5×10^5 in 75-cm³ flasks using high-glucose Dulbecco's modified Eagle's medium with L-glutamine (DMEM) supplemented with 10% fetal bovine serum (FBS; Daiichi Kagaku, Tokyo, Japan), 1% non-essential amino acids (Gibco BRL, Grand Island, NY, USA), 1% penicillin and streptomycin, and 2.5% HEPES. The cells were incubated at 37°C in a humidified incubator with 5% CO₂. Before the required passages of the test were reached, cells were subcultured using trypsin-EDTA solution (0.25%), and the medium was changed every second day.

Bacterial strains and culture conditions

The bacterial strains used in this study were strain LOC1, *L. plantarum* ATCC 14917^T, *L. plantarum* ATCC 8014, *L. plantarum* S206, and *L. plantarum* YT9. *L. plantarum* S206 and *L. plantarum* YT9 were isolated in our laboratory from goat milk and a pickled turnip, respectively. All strains were stored at -80°C in MRS broth and propagated twice in MRS broth prior to use. All strains were grown overnight anaerobically at 37°C in MRS broth. For all studies, the bacterial strains were used in the stationary growth phase.

Preparation of bacterial cells

Bacterial cells from the stationary phase were harvested by centrifugation at 8,000 ×g, followed by two washes with PBS. The bacterial cells were adjusted to a concentration of 1×10^9 cells/mL by counting bacteria with a hemocytometer (Bright-Line, Hausser Scientific, Horsham, PA, USA) and then heat sterilized using an autoclave at 121°C for 15 min. For all experiments, approximately 10^8 heat-sterilized bacterial cells were added to each well.

TEER measurement

Caco-2:HT29-MTX co-cultures (100:0, 90:10, 75:25, and 0:100) were seeded onto 12 mm diameter polyester Transwell inserts with a 0.4 μm² pore size (Corning, Corning, NY, USA) at a density of 1×10^5 cells/well, and DMEM was replaced every second day for 12 days. After the TEER values of the monolayers were measured using a Millicell[®] ERS meter (Millipore, Bedford, MA, USA) connected to a pair of electrodes, which were separately placed in the apical and basolateral compartments, all cells were challenged for 24 hr in a non-supplemented medium (DMEM only, no FBS or antibiotics) or medium supplemented with bacteria (DMEM + bacteria). The TEER values after exposure to bacteria were measured, and all cells were treated with DMEM containing dextran sodium sulfate (DSS; 1% w/v) for 6 hr. After the TEER values were measured again, the differences in the TEER values between before and after the 6 hr incubation were calculated, and the final results were expressed as proportions relative to the initial values. Experiments were repeated three times, with five replicates for each treatment, and the results were expressed relative to the initial TEER values for each insert.

Quantification of mRNA of IEC co-cultures

Expression of mucin- and TJ-related genes in Caco-2:HT29-MTX (90:10 and 75:25) co-cultures was quantified using TaqMan quantitative real-time PCR (qPCR). All reagents were obtained from Applied Biosystems unless otherwise stated.

The expression of these genes in reference samples (untreated controls) was also quantified. The genes quantified were *MUC2*, *MUC4*, *MUC5AC*, *TJP1*, *TJP2*, and *OCN*; their TaqMan assay IDs are Hs.PT.56a.26485553, Hs.PT.56a.5039491, Hs.PT.56a.25473826, Hs.PT.58.39733148, Hs.PT.58.25666947, and Hs.PT.58.24465876, respectively.

Caco-2:HT29-MTX co-cultures (90:10 and 75:25) were seeded into 12-well cell culture plates (Corning) at a density of 1×10^5 cells/well, and DMEM was replaced every second day for 12 days. All cells were challenged for 48 hr in a non-supplemented medium (DMEM only, no FBS or antibiotics) or medium supplemented with bacteria (DMEM + bacteria). After 48 hr, total RNA was extracted from the cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and then isolated using a RNeasy Mini Kit (Qiagen KK, Tokyo, Japan) according to the manufacturer's instructions.

For qPCR analysis, cDNA was synthesized using an iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. cDNA was stored at -20°C prior to determination of the expression levels of the six genes relative to the reference genes hypoxanthine phosphoribosyltransferase [29, 30] (*HPRT1*; Hs.PT.39a 22214821) and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*; Hs.PT.39a 22214836) using TaqMan probes on a QuantStudio 5 Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA). All PCRs were prepared as triplicate 20 μL reactions. The thermal profile used was 95°C for 180 sec followed by 40 cycles of 95°C for 3 sec and 60°C for 30 sec. Cycle threshold values for candidate genes were normalized to the reference genes. Relative expression levels were calculated using the $2^{-\Delta\Delta C_t}$ equation [31]. Experiments were performed in triplicate (three successive passages of cells), each with three replicates per treatment.

Statistical analysis

Data were expressed as mean values with standard variations. Statistical analysis of the data was performed using one-way analysis of variance (ANOVA) followed by Dunnett's test for the TEER value data and Tukey's range test for co-culture data. $P < 0.05$ was considered statistically significant.

RESULTS

Isolation of bacteria from fresh tea leaves

Some colonies were observed after the solution containing ground fresh tea leaves was plated onto MRS agar supplemented with sodium carbonate and incubated. They were opaque and shiny milky white colonies with neat edges and a diameter of 2–3 mm. One of the isolates, designated strain LOC1, was randomly selected and subjected to PCR for confirmation of its genus and species with genus-specific and species-specific primers, respectively. PCR analysis using primer pairs targeting *Lactobacillus* confirmed that strain LOC1 was *Lactiplantibacillus* (Fig. 1). Therefore, because *L. plantarum* has previously been found in plant materials [32–34], a subsequent PCR analysis was performed using primer pairs targeting *L. plantarum*, and it suggested that strain LOC1 was *L. plantarum* (Fig. 2).

To validate the results obtained from PCR analysis, the *pheS* gene sequence was determined (Table 2) and compared with those in the NCBI database (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). The *PheS* gene sequence showed a similarity of more than

99% to the other *L. plantarum* strains in the GenBank database, demonstrating that strain LOC1 belonged to *L. plantarum*.

Strain LOC1 suppresses DSS-induced decreases in TEER

The TEER value is an important parameter used to study intestinal barrier integrity. The initial TEER values (before DSS treatment) of the 100:0, 90:10, 75:25, and 0:100 Caco-2:HT29-MTX cultures varied from 470–510 $\Omega \times \text{cm}^2$, 440–480 $\Omega \times \text{cm}^2$, 340–370 $\Omega \times \text{cm}^2$, and 100–140 $\Omega \times \text{cm}^2$, respectively.

DSS, a polysaccharide similar to heparin, contains approximately 17% sulfur and includes up to three sulfates per glucose molecule [35]. It has been used as a model for experimentally induced colitis and colon cancer [36] and to decrease the TEER of intestinal epithelial cells [37]. After 6 hr of exposure to DSS, the TEER values of all co-cultures decreased (data not shown). Meanwhile, strain LOC1, but not *L. plantarum* ATCC 14917^T and *L. plantarum* ATCC 8014, significantly suppressed the DSS-induced decrease in TEER values of

the Caco-2:HT29-MTX 100:0 and 90:10 cultures ($p < 0.05$; Fig. 3a and 3b). The 75:25 and 0:100 co-cultures showed no significant suppression of the DSS-induced decrease in TEER values (Fig. 3c and 3d). These results suggested the beneficial effects of strain LOC1 on the intestinal barrier integrity.

Strain LOC1 impacts on TJ-related gene expression

To investigate the effects of strain LOC1 on the intestinal barrier integrity in detail, the ability of strain LOC1 to alter the expression levels of three TJ-related genes in Caco-2:HT29-MTX co-cultures (90:10 and 75:25) was compared with those of 4 *L. plantarum* strains using qPCR. In both 90:10 co-cultures and 75:25 co-cultures, the mRNA expression of OCLN was significantly increased after exposure to strain LOC1 and *L. plantarum* S206 compared with controls ($p < 0.01$; Fig. 4a, b). However, the mRNA expression of TJP1 and TJP2 showed no significant effect of bacteria.

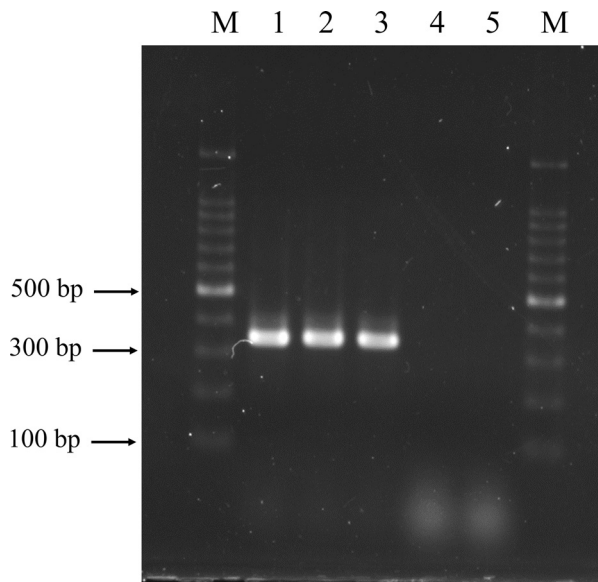


Fig. 1. Agarose gel for PCR products amplified by genus-specific primers of *Lactobacillus*. Lanes: M, 100 bp DNA ladder; 1, strain LOC1; 2, *L. plantarum* ATCC 14917^T; 3, *L. plantarum* ATCC 8014; 4, *Bifidobacterium lactis* Bb12; 5, distilled water.

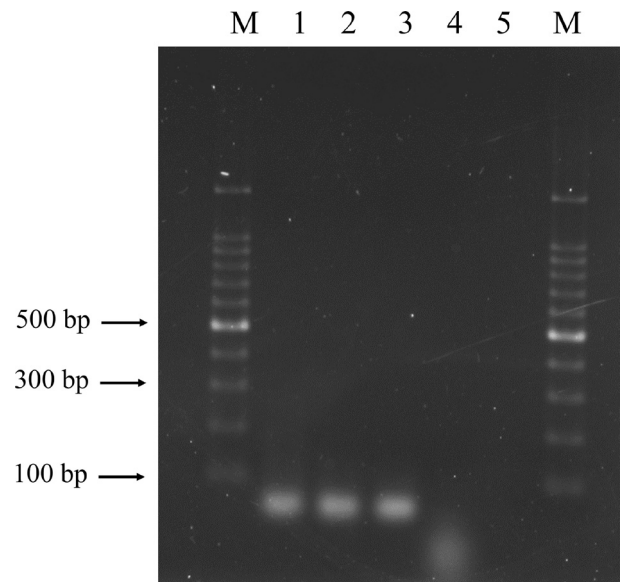


Fig. 2. Agarose gel for PCR products amplified by species-specific primers of *L. plantarum*. Lanes: M, 100 bp DNA ladder; 1, strain LOC1; 2, *L. plantarum* ATCC 14917^T; 3, *L. plantarum* ATCC 8014; 4, *L. acidophilus* La5; 5, distilled water.

Table 2. Base sequences of phenylalanyl-tRNA synthase alpha subunit (*pheS*)

Gene	Base sequence
<i>pheS</i>	ATGAGTTTACAAGATCGATTAACCGAATTACGCGATCAAGGCTTGGCCGATATTAATCCGCCGATGTTTTGAAAAAGGTTAAC CAAGTCAAAGTTGATTTACTTGGCAAAAAGGGTCCGATTACAGAAGTATTGCGCGAATGCGCGACTTAAGCCCGGAAGAACG GCCAAAGGTGGGCGCTTATGCCAACGAAGTTCGTGACCGGATTACAGGCTGCGATTGATGAACGCCGTGAAGAAGTGAACAA GCAGCCGTTAATGAGCAATTGGCTGCCGAAAACTGGACGTGACGTTACCGGGTCGGAAGTTCACAAGGTCAGCCTCAG TGATTACCCAGATTACTGAATTGGAAGATCTATTTATGGGAATGGGCTATCAAATGTTGATGGTGATGAAGTTGAAGAAGA TTACTACAACCTTTGAACGGTTGAACCTTACCGAAGGACCATCCCGCCCGTGACATGCAAGACACGTTCTATATTACCAAAGACGT GCTACTACGCACGCAGACGTCTGCTGATCAGCCGCGGTCACTTAAAAATCACGATTTTCTAAAGGACCGCTGAAGGCTTTGTC ACCTGGCCGCGTTTATCGGCGTGATACGGATGATGCAACCCATCCCATCAATTCATCAAATTGAAGGGTTAGTCGTGGACAAG CATATTACGATGGCTGATTTGAAGGGCACCTTAATTCTGGTTGCCAAGACTTTGTTGGCGATCAATTCGATGTTCCGGCTACGGCC AAGCTTCTTTCCATTACGGAACCATCCGTAGAAGCTGATGTAACCTGCTTTAATTGCAATGGCAAGGGCTGTGCAATCTGTAAG CAAACGGGTTGGATCGAAGTACTGGGTGCCGGCATGGTTCACCCCCACGTGTTAGAAATGTCTGGCATTGATCCAGAAGAATAT GGTGGTTTTGCCTTTGGGTTAGGACCAGACCGCTTTGCAATGTTGAAATACGGTGTGACGATATCCGCAACTTCTACTTGAATG ACGTGCGGTTCTTGTACAGTTCTATAAGAAAGGTTAG

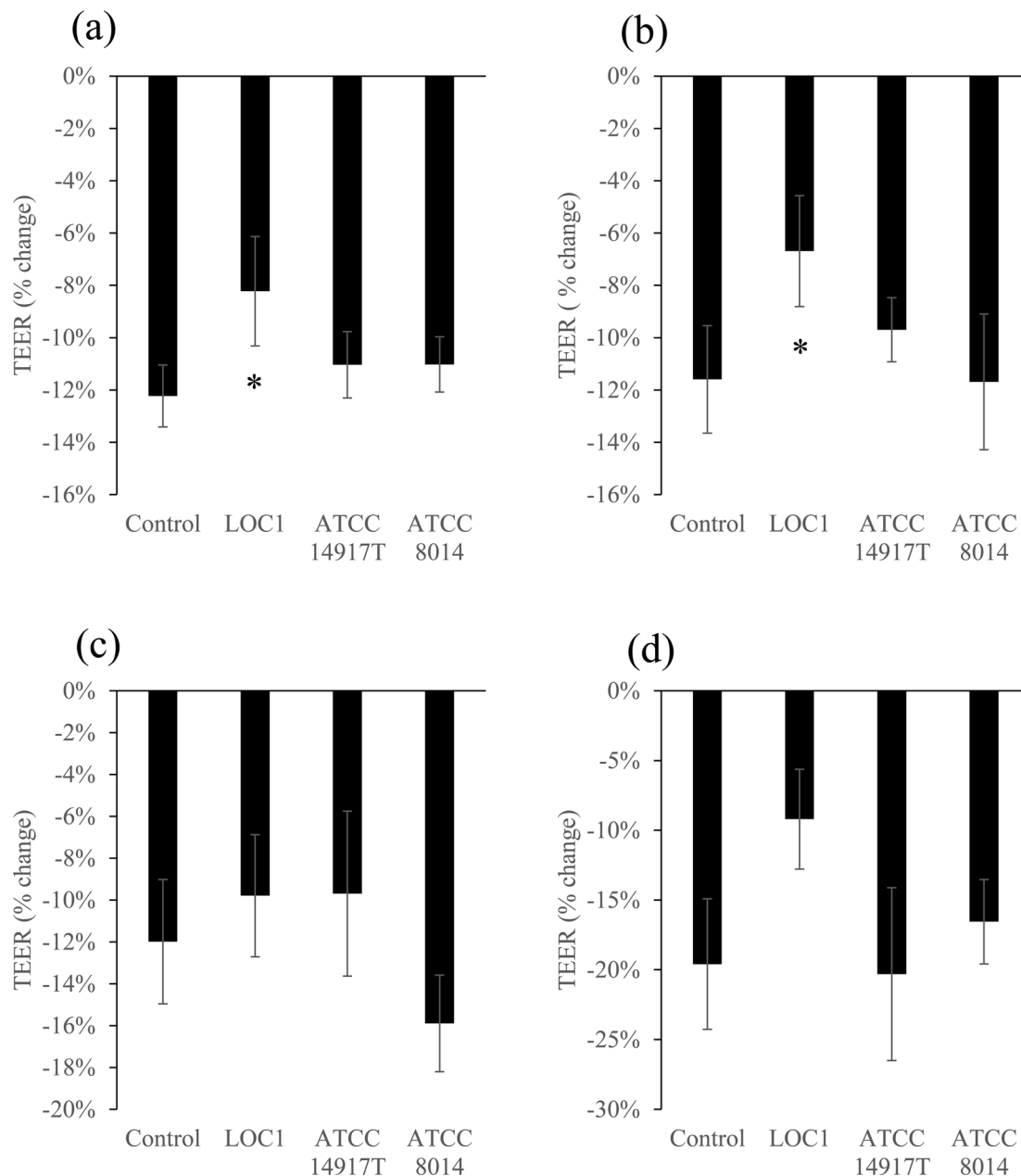


Fig. 3. Influence of *L. plantarum* on the transepithelial resistance (TEER) of 100:0 (a), 90:10 (b), 75:25 (c), and 0:100 (d) Caco-2:HT29-MTX co-cultures. All cells were challenged for 24 hr in a non-supplemented medium (DMEM only, no FBS or antibiotics) or medium supplemented with bacteria (DMEM + bacteria). The TEER values after bacterial treatment were measured. All cells were treated with DMEM containing dextran sodium sulfate (DSS; 1% w/v) for 6 hr. Changes in TEER are shown as the percentage change after 6 hr compared with the initial TEER. Values are means (\pm SEM) of three experiments (n=3; three samples per treatment per experiment). Control, untreated; LOC1, strain LOC1; ATCC14917, *L. plantarum* ATCC 14917^T; ATCC8014, *L. plantarum* ATCC 8014. *Significant differences between each treatment and the control with no treatment (p<0.05).

Strain LOC1 impacts on mucin expression

Mucins secreted by goblet cells are an important aspect of the protective capacity of the intestinal barrier. They form a mucus layer with water and cover the epithelial free surface, providing lubrication and antagonizing the intestinal adhesion and invasion of pathogenic bacteria [38]. Thus, the effect of bacteria on the expression of MUC2, MUC4, and MUC5AC was assessed through a comparison with four *L. plantarum* strains. In 90:10 co-cultures, the mRNA expression levels of MUC2 and MUC4 were

significantly upregulated (p<0.05) after exposure to strain LOC1, *L. plantarum* ATCC 8014, and *L. plantarum* S206 compared with controls (Fig. 5a). Furthermore, the result was the same even in the case of 75:25 co-cultures (Fig. 5b). No significant changes in MUC5AC mRNA expression were seen in any of the co-cultures after treatment with bacteria, compared with controls.

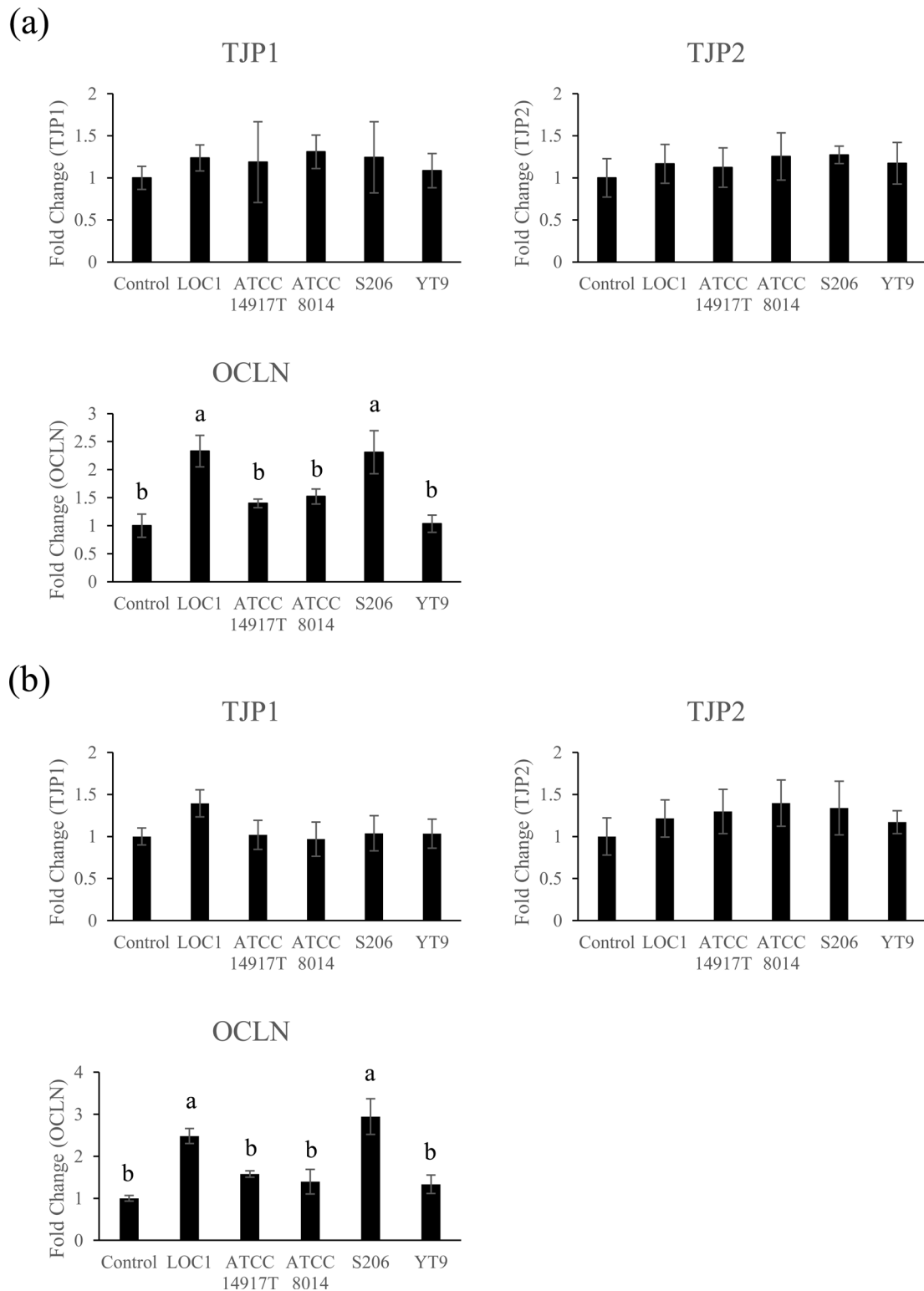


Fig. 4. Fold changes of TJP1, TJP2, and OCLN mRNA in 90:10 (a) and 75:25 (b) Caco-2:HT29-MTX co-cultures after 48 hr incubation with bacteria. Data are expressed as the mean fold changes (\pm SEM) of three replicates across three independent experiments ($n=3$). Control, untreated; LOC1, strain LOC1; ATCC14917, *L. plantarum* ATCC 14917^T; ATCC8014, *L. plantarum* ATCC 8014; S206, *L. plantarum* S206; YT9, *L. plantarum* YT9. Different letters (a and b) indicate a significant difference between samples ($p<0.05$).

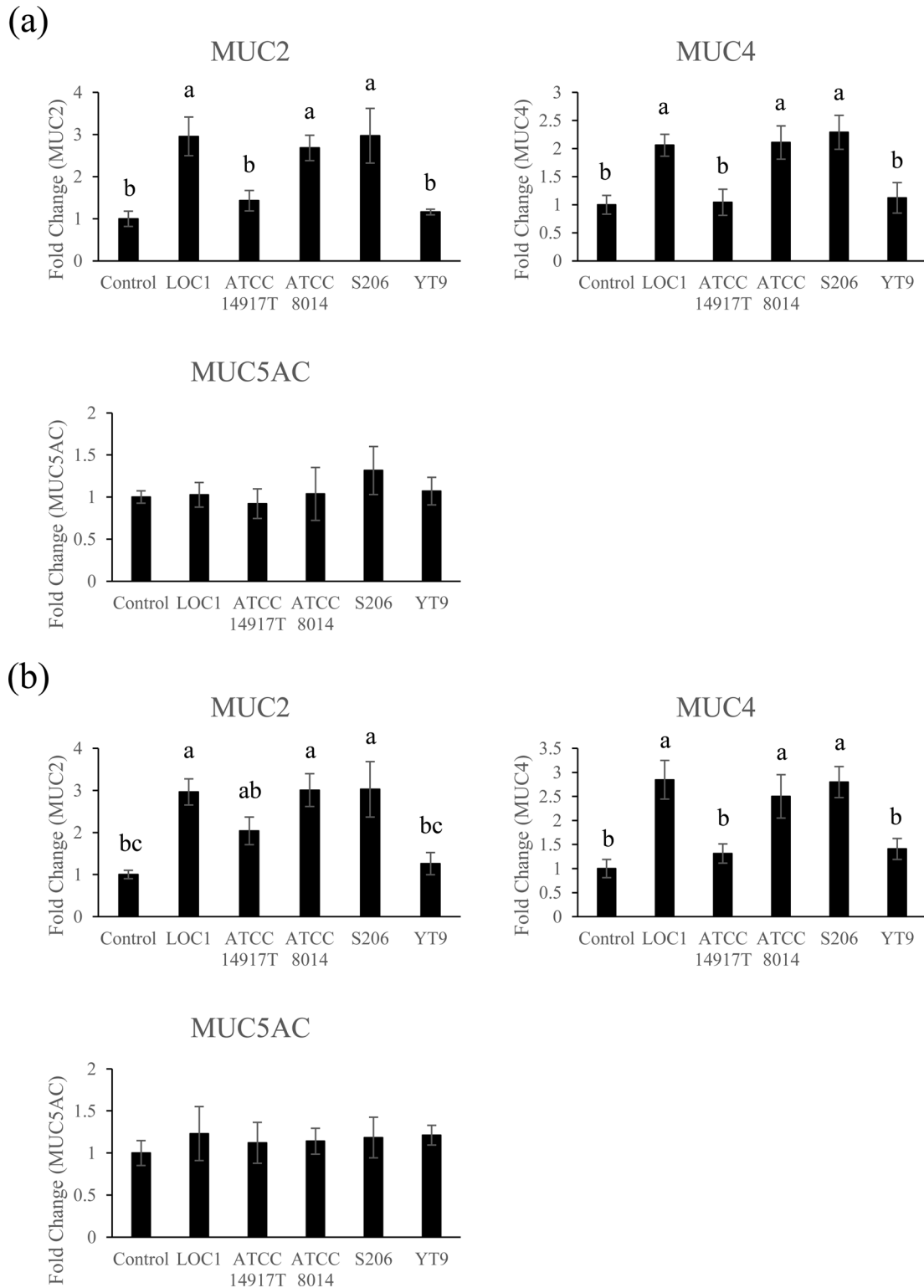


Fig. 5. Fold changes of MUC2, MUC5AC, and MUC4 mRNA in 90:10 (a) and 75:25 (b) Caco-2:HT29-MTX co-cultures after 48 hr incubation with bacteria. Data are expressed as the mean fold changes (\pm SEM) of three replicates across three independent experiments ($n=3$). Control, untreated; LOC1, strain LOC1; ATCC14917, *L. plantarum* ATCC 14917^T; ATCC8014, *L. plantarum* ATCC 8014; S206, *L. plantarum* S206; YT9, *L. plantarum* YT9. Different letters (a, b, and c) indicate a significant difference between samples ($p<0.05$).

DISCUSSION

Strain LOC1 isolated from fresh tea leaves was identified as *L. plantarum* by PCR and *pheS* sequence analysis. *L. plantarum* is present in plant materials and the gastrointestinal tracts of animals, and it has been indicated by some reports to be a probiotic species of lactic acid bacteria [32–34]. For example, some *L. plantarum* strains alleviate irritable bowel syndrome [39, 40]. *L. plantarum* JSA22 has inhibitory activity against *Salmonella enterica* subspecies *enterica* serovar Typhimurium infection of intestinal epithelial cells, while *L. plantarum* CICC23174 has immune stimulatory activity, such as by enhancing the phagocytic activity of macrophages [33]. Furthermore, *L. plantarum* has the ability to reduce soy allergy [41]. It is also known to produce bacteriocins [42, 43]. For example, plantaricin KL-1Y derived from *L. plantarum* KL-1 has not only sterilization action against *Bacillus cereus* but also inhibitory actions against the growth of *Listeria innocua*, *Staphylococcus aureus*, and *Escherichia coli* [44]. It has also been shown that extracellular proteins derived from *L. plantarum* BMCM 12 inhibit the adhesion of *E. coli* and *S. enterica* subsp. *enterica* to mucin [45]. These reports show that *L. plantarum* is a member of the beneficial species of LAB that are used as probiotics. We have shown that the enhancing effect of strain LOC1 on barrier integrity was greater than those of *L. plantarum* ATCC 14917^T and *L. plantarum* YT9, which suggests that fresh tea leaves could be utilized as a source for isolating probiotics.

After heat treatment, important probiotic properties are retained in industrially grown probiotic bacteria, including bacterial extracts and supernatants in most cases, so it is possible to develop safer preparations with more optimal pharmaceutical properties (such as a long shelf life) [46–48]. In addition, various strains of lactic acid bacteria and bifidobacteria can produce beneficial effects in their heat-inactivated forms [49]. For instance, heat sterilization has shown efficacy in maintaining the integrity of the intestinal barrier. Heat-sterilized *Lactocaseibacillus rhamnosus* OLL2838 has been reported to protect against colitis-induced mucosal barrier permeability defects in mice [50]. In Caco-2/TC7 cell monolayers, heat-sterilized *L. acidophilus* LB and its culture medium offset the increase in paracellular permeability induced by infection with diffusely adhering *E. coli* C1845 expressing the Afa/Dr adhesins [51]. This study showed the beneficial contribution to the intestinal barrier of strain LOC1, which was heat-killed using an autoclave, suggesting that strain LOC1 could be used as a safe probiotic.

TEER values were used to assess intestinal barrier integrity. Our results indicated that strain LOC1 suppressed DSS-induced decreases in TEER in Caco-2:HT29-MTX 100:0 and 75:25 co-cultures, which suggests that strain LOC1 plays an important role in changes in intestinal cell permeability. It is well known that changes in intestinal permeability are a major contributor to predisposition to intestinal inflammatory diseases and diarrhea [52]. To fully evaluate the impact of strain LOC1, cell viability assays may be needed to assess intestinal epithelial cell activity after exposure to strain LOC1.

In Caco-2:HT29-MTX co-cultures (90:10 and 75:25), OCLN mRNA expression was significantly increased after exposure to strain LOC1 compared with controls. Increased expression or abundance of occludin is related to protection of the epithelial barrier, whilst reduction of occludin levels is related to epithelial

barrier dysfunction and increased epithelial permeability [53]. Conversely, the levels of TJP1 and TJP2 mRNA did not change as a result of any treatment. This suggests that the DSS-induced reduction in TEER values may have been due to changes in other TJ components not measured in this study. For example, an increase in the TEER of a Caco-2 monolayer after treatment with *L. plantarum* MB452 was found to be due to cingulin, which is an intracellular plaque protein [54] that binds directly to not only TJP1 but also actin filaments of the cytoskeleton [12]. Claudin 1, which is a transmembrane TJ protein and interacts directly with TJP1, was upregulated in the jejunal epithelium of young piglets after treatment with *Limosilactobacillus reuteri* I5007 [55]. Probiotic bacteria can enhance the intestinal barrier through various mechanisms. Therefore, the mechanism by which a heat-killed strain LOC1 fraction can exert its effects needs further research.

The mucus layer of the gastrointestinal tract performs a first-line function in the defense against threats such as mycotoxins and provides a beneficial environment for the endogenous symbiotic microflora [56]. It has also been confirmed that the presence or absence of mucin secreted by goblet cells in the gastrointestinal tract is associated with not only gastrointestinal inflammation and related diseases but also cancer [57, 58]. Co-cultures incubated with strain LOC1, *L. plantarum* ATCC 8014, and *L. plantarum* S206 had significantly increased levels of both MUC2 and MUC4 mRNA compared with control co-cultures. This suggests that these strains play an important role in promoting mucin secretion. MUC2 is a major secreted glycoprotein that is abundantly expressed by the intestinal and airway epithelium [59]. Its expression is a common feature of all mucinous cancers derived from various organs, such as breast, colon, and prostate cancers, so it may serve as a potential prognostic indicator [60–62]. As the major components of the glycocalyx, membrane-bound mucins such as MUC4 are involved in a wide range of interactions in the luminal environment [63] (such as intracellular signaling events [64]). They are also useful in fetal development, epithelial regeneration and differentiation, and epithelial integrity [65, 66].

Conversely, no significant changes in MUC5AC mRNA expression were seen after treatment with bacteria. Unlike HT29-MTX cells, the Caco-2 monolayer does not show the full function of goblet cells capable of secreting mucin [67, 68]. Thus, in Caco-2: HT29-MTX co-cultures (90:10 and 75:25), MUC5AC mRNA expression was very low, and this could have been a cause of this result.

Based on the results obtained in this study, it can be concluded that *L. plantarum* LOC1 isolated from fresh tea leaves could be used as a safe probiotic with beneficial effects on the intestinal barrier. Few studies have examined the isolation of *Lactobacillus* from fresh tea leaves and the beneficial effects of this strain. Therefore, to the best of our knowledge, this is the first report of the isolation, identification, and impact on intestinal barrier integrity of *L. plantarum* from fresh tea leaves. In the future, in vitro and in vivo studies for elucidating the precise mechanisms will be needed to confirm the benefits and probiotic potential of strain LOC1.

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