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Research Article

Characterization of *Lactiplantibacillus plantarum* TO-A Growth Inhibition Activity against Pathogenic Bacteria.

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ABSTRACT

Lactiplantibacillus plantarum is commonly used in pharmaceuticals and foodstuffs. Some *L. plantarum* strains are known as lactic acid bacteria that can inhibit the growth of pathogenic bacteria. A significant reclassification of the genus *Lactobacillus* was carried out in 2020. However, *L. plantarum* TO-A, which was isolated and registered in 1997, has not been sufficiently characterized. Therefore, in this study, we conducted a taxonomic re-identification of *L. plantarum* TO-A and judged that it should be classified as *L. plantarum* subsp. *plantarum*. Using *in vitro* experiments, we also determined that *L. plantarum* TO-A has a higher lactic acid production capability than other lactic acid bacteria (*L. plantarum* ATCC14917, *L. reuteri* NBRC15892, *L. gasseri* ATCC19992, and *L. rhamnosus* ATCC53103) and that *L. plantarum* TO-A inhibits the proliferation of four pathogenic bacteria (*Escherichia coli* ATCC8739, methicillin-resistant *Staphylococcus aureus* ATCC33591, *Clostridium perfringens* ATCC 13124, and *Clostridium difficile* ATCC17859). In parallel experiments, we used *Caenorhabditis elegans* to confirm that *L. plantarum* TO-A prevents bacterial infection of the host *in vivo*. Consequently, compared with nematodes fed *E. coli* OP50, nematodes fed *L. plantarum* TO-A exhibited longer survival in the presence of methicillin-resistant *S. aureus*. Moreover, in *in vitro* experiments, we showed that *L. plantarum* TO-A eliminated up to 65.3 % of mucin adherent methicillin-resistant *S. aureus*. Our study thus suggests that *L. plantarum* TO-A used in pharmaceuticals and foodstuffs can help protect the host from various pathogenic bacteria.

Keywords: Probiotics; lactic acid bacteria; *Lactiplantibacillus plantarum*; growth inhibition activity; *Caenorhabditis elegans*.

INTRODUCTION

The term "probiotics" is derived from the Latin and Greek word "pro bios", which means "for life". Since probiotics was defined by A. Fuller in 1989, its meaning has been revised with emerging data, and it is now defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" [1,2]. Numerous studies have demonstrated that probiotic bacteria regulate cytoplasmic pH, inhibit the proliferation and colonization of pathogenic bacteria, and regulate the host immune response [3-5]. Thus, in recent years, probiotic bacteria have received considerable research attention as therapeutic agents for treating various diseases.

Lactic acid bacteria (LAB) are representative species used in probiotic preparations. *Lactiplantibacillus plantarum* in particular is one of the most commonly employed LAB species in probiotic preparations. *L. plantarum* is a gram-positive microaerophilic rod included in the heterofermentative group and produces both L-and D-lactic acid [6]. Rigorous study of *L. plantarum* has proceeded over many years, and the ability of *L. plantarum* to inhibit the growth of pathogenic bacteria has been widely reported. For example, by Nwachukwu and colleagues reported that an inhibition circle is formed on the bacterial lawn when pathogenic bacteria are applied to an agar plate on which *L. plantarum* is cultured [7]. Moreover, Danilova and colleagues reported that *L. plantarum* culture supernatant inhibits the proliferation of pathogenic bacteria and suggested that this inhibition effect is associated with metabolites released by *L. plantarum* [8]. In addition, *in vitro* experiments conducted by Mukherjee and Ramesh revealed that *L. plantarum* inhibits the adhesion of *Staphylococcus aureus* to collagen and mucin [9]. Thus, many reports suggest that *L. plantarum* inhibits the proliferation of various pathogenic bacteria via the release of certain metabolites. However, the abovementioned cases could indicate the activity of specific strain; thus, these data are not necessarily applicable to all strains [10].

L. plantarum TO-A (LP TO-A) was isolated from silage and identified using 16s rRNA sequences (accession number: LC651778) and registered with the NITE Microorganisms Depository in 1997 (deposit number: FERM P-16564). However, taxonomic verification of this organism has been not carried out, despite its continued use in functional foods. In addition, *Lactobacillus plantarum* was reclassified into the newly proposed genus *Lactiplantibacillus* in 2020 by Zheng and colleagues [11]. Recently, the need for a taxonomic re-

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identification of LP TO-A has increased. Hence, we attempted a taxonomic re-identification of LP TO-A in this study. Moreover, to demonstrate the effectiveness LP TO-A as a probiotic bacterium, we conducted a parallel investigation to determine whether LP TO-A exhibits the above-reported characteristics. *Caenorhabditis elegans*, a nematode bacterivore usually fed the single bacterium (*Escherichia coli* OP50) in the laboratory, was used in these experiments. By employing treatment with hypochlorite solution, the intestinal microbiome of *C. elegans* can be easily controlled. *C. elegans* are ideally suited to whole-life survival assays because their lifespan is relatively short, at 2-3 weeks [12]. Hence, this nematode species is often used to study interactions between probiotic bacteria and the host [13]. Moreover, *C. elegans* is a useful host model for studying bacterial pathogenesis and screening antibacterial substances [14,15]. In this study, we investigated the growth inhibition activity of LP TO-A *in vivo* and examined whether pre-feeding nematodes LP TO-A mitigates the effects of bacterial infection.

MATERIALS AND METHODS

Bacterial strains and growth conditions

The following bacterial strains were used in this study: *Lactiplantibacillus plantarum* TO-A (LP TO-A), *Lactiplantibacillus plantarum* subsp. *plantarum* ATCC14917 (type strain), *Lactiplantibacillus plantarum* subsp. *plantarum* NBRC15891, *Lactiplantibacillus plantarum* subsp. *argentoratensis* NBRC106468, *Lactiplantibacillus paraplantarum* JCM12533, *Lactiplantibacillus pentosus* NBRC106467, *Limosilactobacillus reuteri* NBRC15892, *Lactobacillus gasseri* ATCC19992, *Lacticaseibacillus rhamnosus* ATCC53103, *Escherichia coli* ATCC8739, *Escherichia coli* OP50, methicillin-resistant *Staphylococcus aureus* ATCC33591 (MRSA), *Clostridium perfringens* ATCC13124, and *Clostridium difficile* ATCC17859. LAB were grown in 5 ml of *Lactobacillus* medium according to DeMan, Rogosa and Sharpe (MRS, Difco, USA) broth under anaerobic conditions at 37 ˚C. Four strains of pathogenic bacteria (*Escherichia coli* ATCC8739, MRSA, *Clostridium difficile* ATCC17859, and *Clostridium perfringens* ATCC13124) were grown in 5 ml of Brain Heart Infusion (BHI, Eiken, Japan) broth (including 1 % glucose) under anaerobic conditions at 37 ˚C. Moreover, *E. coli* OP50 was grown in 5 ml of Luria-Bertani (LB) broth at 37 ˚C.

Taxonomical identification of LP TO-A

Multi-locus phylogenetic analysis was performed using the *rec*A and *cpn*60 genes. Genomic DNA of *Lactiplantibacillus* species was extracted as described by Saito and Miura (1963), Boom and colleagues (1990) and Sato and colleagues (2014), with some modifications [16-18]. Briefly, appropriate aliquots of bacteria were suspended in lysis buffer and homogenized three times using a FastPrep FP120 disruptor (MP Biomedicals, USA) operated at 6.5 m/sec for 20 sec, and the resulting lysate was subjected to phenol extraction and ethanol precipitation of DNA. The precipitated DNA was purified using a Monofas DNA purification kit (Animos, Japan) according to the manufacturer's instructions. Finally, the DNA was eluted in TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]). For amplification of the *rec*A and *cpn*60 gene regions, PCR was performed using a KOD-plus neo (Toyobo, Japan) and CFX96 Real-Time System (BioRad, USA) with the primer set *rec*A (forward: 5'-AGAAACGAGCACCAAAGGAG-3'; reverse: 5'-AGTGTCAACTTAAAGTCTTATGGCAAC-3'), and the primer set *cpn*60 (forward [upstream]: 5'-TTTAGATAACGGGACAAAAGTTGAC-3'; forward [downstream]: 5'-GCACACGAAGTTAAGACGCAAGAAG-3'; reverse: 5'-TTGCGTTAACGACTAATCCTACC-3'), respectively. The amplification program consisted of 1 cycle of 94 ˚C for 2 min, 30 cycles of 98 ˚C for 10 sec, 69 ˚C for 30 sec, and 68 ˚C for 1.5 min, followed by 1 cycle of 68 ˚C for 7 min. Sequence analysis of the resulting products was outsourced to Fasmac Co., Ltd (Japan). Catalase and oxidase tests were performed as described in microbiology: A laboratory manual [19]. Editing, assembly, and alignment of the DNA sequences and subsequent generation of a phylogenetic tree were performed using CLC genomics workbench, ver. 21 (Qiagen, Germany). For multi-locus phylogenetic analysis, the homologous nucleotide sequence data were obtained from GenBank. The nucleotide sequence alignment of concatenated genes consisted of approximately 2600 bp (1100 bp from *rec*A and 1600 bp from *cpn*60), and the reliability of internal branches of the phylogenetic tree was assessed from 100 bootstrap pseudo-replicates. The GenBank/EMBL/DDBJ accession numbers for the *rec*A and *cpn*60 sequences generated in this study were LC651779 to LC651783 (*rec*A partial gene sequences) and LC651784 to LC651788 (*cpn*60 partial sequences). Additionally, for physiologic and phenotypic characterization of LP TO-A, biochemical characteristics regarding the production of acid from various carbohydrates by *Lactiplantibacillus* species were recorded after 2 days of incubation using an API 50 CH system (BioMe´rieux, France), following the manufacturer's suggested protocol.

Detection of D/L-lactic acid in culture supernatant

Using a 0.45-μm PVDF membrane filter, the supernatant was collected from MRS broth in which LAB were cultured for 2 days. The concentrations of D/L-lactic acid in the supernatant were investigated using an enzymatic bioanalysis kit (F-kit D/L-lactic acid, Roche, Switzerland). This experiment was performed according to the method described in the product attachment. Nicotinamide adenine dinucleotide (NADH) was formed in reactions between D/L-lactic acid and D/L-lactate dehydrogenase, and the amount of NADH indicated the amount of D/L-lactic acid in the sample. NADH was determined spectrophotometrically because it exhibits an absorption peak at 339 nm. Therefore, the amount of D/L-lactic acid in the supernatant was calculated based on the absorbance of NADH using a calibration curve for the relationship between the absorbance of NADH and amount of D/L-lactic acid. The absorbance of NADH was determined spectrophotometrically (wavelength: 340 nm, GeneQuont100, GE Healthcare, USA).

Assay of LAB growth

LAB cultured for 1 day were prepared to an optical density at "660 nm" (OD_{660}) of 1.0, after which 100 μl of prepared solution and 100 μl MRS broth were added to each well of a 96-well plate. Next 50 μl of liquid paraffin was added to each well in order to prevent medium evaporation. Under anaerobic conditions, the absorbance (OD_{660}) was measured every 30 min for 18 h using a microtiter plate reader (Sunrise Rainbow Thermo RC, Tecan, Switzerland).

Growth inhibition assay

LAB were spread to the centre of a BHI (including 1 % glucose) plate using a glass rod and cultured for 2-3 days. Pathogenic bacteria were then spread around the lawn of LAB and cultured for 3 days. The inhibition circle was using an E-P2 camera (OLYMPUS, Japan). The supernatant of LAB cultured for 2 days was collected by removing bacteria using a 0.45-μm PVDF membrane filter. Pathogenic bacteria antecedently cultured for 1 day were prepared to OD_{660} 1.0, and 100 μl of prepared culture was added to each well of a 96-well plate.

Next, 75 µl of BHI broth (including 1 % glucose) and 25 µl of LAB culture supernatant were mixed and added to the wells. Moreover, to prevent medium evaporation, 50 µl of liquid paraffin was added to each well. Under anaerobic conditions, the absorbance (OD_{660}) was measured every 30 min for 16 h using a microtiter plate reader (Sunrise Rainbow Thermo RC, Tecan, Switzerland).

Assay of LAB adhesion

The assay was conducted with reference to previous reports [9,20,21]. First, 1 mg/ml mucin (porcine stomach, FUJIFILM Wako, Japan) was prepared in 0.1 M acetate buffer (pH 5.0). Next, 3 mg/ml collagen (Cellmatrix type I-P, Nitta Gelatin, Japan) was diluted 11-fold with hydrochloric acid buffer (pH 3.0). Finally, 100 µl of collagen or mucin solution was added to each well of 96-well immune-plate (Maxisorp Nunc, Thermo Scientific, USA) and incubated overnight at 4 °C. After washing three times with 100 µl of PBS, the coated wells were blocked whit 100 µl of PBS (including 1 % Tween 20) for 1 h and washed twice with 100 µl of PBS. LAB culture for 1 day were washed with PBS buffer and labelled under anaerobic conditions for 20 min at 37 °C with 50 µM cFDA-SE. Subsequently, using PBS, labelled bacteria were washed and suspended to OD_{660} 1.0, after which 100 µl of bacterial solution was added to each well of the 96-well immune-plate, and cultured under anaerobic conditions for 2 h at 37 °C. After washing three times with PBS, 100 µl of PBS was added to each well of the 96-well immune-plate, and the fluorescence intensity was measured (Ex: 492 nm, Em: 540 nm) using a multi-hybrid plate reader (Spark, Tecan, Switzerland). The adherence rate was calculated using the following formula:

Adherence rate=([Fluorescence intensity# of adhered cells − # of PBS buffer] / [# of total cells − #of PBS buffer])×100.

(Fluorescence intensity = #)

Adherence inhibition assay

The assay was conducted with reference to a previous report by Mukherjee and Ramesh [9]. MRSA cultured for 1 day were washed with PBS and labeled under anaerobic conditions for 20 min at 37 °C with 50 µM cFDA-SE. The labeled MRSA cells were washed with PBS and then resuspended to OD_{660} 1.0. To investigate the ability of LAB to inhibit the adherence of MRSA cells, 100 µl labeled MRSA was added to the coated wells of a 96-well immune-plate and allowed to stand for 1 h. The MRSA solution was then removed, and the wells were washed with PBS. Next, 100 µl of LAB adjusted to OD_{660} 1.0 with PBS was added to the washed wells and allowed to stand for 1 h. After washing three times with PBS, 100 µl of PBS was added to each well of the 96-well immune-plate, and the fluorescence intensity was measured (Ex: 492 nm, Em: 540 nm) using a multi-hybrid plate reader (Spark, Tecan, Switzerland). The rate of MRSA adhesion was calculated using the same formula described above in "Assay of LAB adhesion".

Caenorhabditis elegans growth conditions

Caenorhabditis elegans N_2 Bristol was cultured at 20 °C on *E. coli* OP50 cells in nematode growth medium (NGM). To synchronize *C. elegans*, 10 adult nematodes were transferred to fresh NGM plates and removed after 4 h, which eggs were cultured on fresh NGM plates at 20 °C for 3 days.

Survival assay

Bacterial solution (LP TO-A, *E. coli* OP50, or MRSA) was prepared to OD_{660} of 1.0 with LB, MRS, or BHI (including 1 % glucose) broth, and 200 µl of respective bacterial solution was spread over the entire surface of a 35 mm plate, which was incubated for 24 h at 37 °C. Synchronized adult nematodes were collected using M9 buffer and washed three times. A total of 20-30 nematodes were transferred to the LP TO-A plate or *E. coli* plate and cultured for 1 day at 20 °C, after which 20 nematodes were transferred to the MRSA plate and monitored at 20 °C. The nematodes were transferred to a fresh MRSA plate each day and considered dead when they exhibited no pharyngeal pumping and had no reaction to prodding with a picker. Nematodes that died as a result of climbing the wall of the plate were not included in the assay. The experiments were performed three times, and a total of more than 300 nematodes were used in the experiments.

RESULTS

Taxonomic re-identification of LP TO-A

Multi-locus phylogenetic analysis results are depicted as a phylogenetic tree reconstructed from the concatenated sequences of two protein-coding gene loci (*rec*A and *cpn*60) (Figure 1). At the interspecies level, all *Lactiplantibacillus* species were clearly differentiated and occupied distinct clusters. Moreover, the tree revealed two sub-clusters within the *Lactiplantibacillus plantarum* group (*L. plantarum* subsp. *plantarum* and *L. plantarum* subsp. *argentoratensis*), and LP TO-A belonged to the *L. plantarum* subsp. *plantarum* cluster. The closest neighbor of *L. plantarum* group was *L. paraplantarum*; *L. pentosus* was more distantly related to these species. We also determined the carbohydrate fermentation patterns of the *Lactiplantibacillus* strains using API 50 CH (Tables 1 and S1). *L. plantarum* ATCC14917, LP TO-A, *L. paraplantarum* JCM12533, and *L. argentoratensis* NBRC106468 were all classified as *Lactobacillus plantarum* 1, and only *L. pentosus* NBRC106467 was classified at *Lactobacillus pentosus*. The average identification scores calculated using the API WEB site were 99.9 % for *L. plantarum* ATCC14917 and LP TO-A, but only 86.6 % and 60.2 % for *L. paraplantarum* JCM12533 and *L. argentoratensis* NBRC106468, respectively. These results suggest that LP TO-A

Table 1: Morphological, physiological, and fermentative characteristics of *Lactiplantibacillus* strains.

	L.plantarum TO-A	*L.plantarum* ATCC14917	*L.paraplantarum* JCM12533	*L. argentoratensis* NBRC106468	*L. pentosus* NBRC106467
Gram stain	+	+	+	+	+
Shape	rod	rod	rod	rod	rod
Spore forming	-	-	-	-	-
Motility	-	-	-	-	-

Facultative anaerobe	+	+	+	+	+
Catalase	-	-	-	-	-
Oxidase	-	-	-	-	-
Identification of API 50CH (% Avg. ID)	*Lactobacillus plantarum* 1 (99.9 %)	*Lactobacillus plantarum* 1 (99.9 %)	*Lactobacillus plantarum* 1 (86.6 %)	*Lactobacillus plantarum* 1 (60.2 %)	*Lactobacillus* pentosus (99.9 %)

+: positive; -: negative; % Avg. ID: average identification score (indicated by APIWEB [https://apiweb.biomerieux.com]).

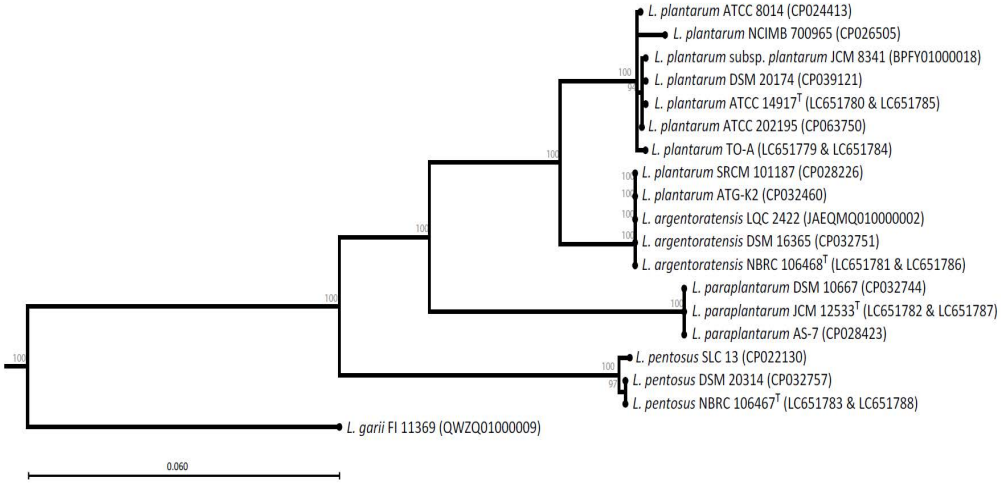

Figure 1: Multilocus phylogenetic tree based on the *rec*A and *cpn*60 gene sequences.
Tree showing the relative positions of *L. plantarum* subsp. *plantarum*, *L. plantarum* subsp. *argentoratensis* and other *Lactiplantibacillus* strains as inferred using the neighbor-joining method. *L. garii* was used as the outgroup taxon to root the tree. Bootstrap values for a total of 100 replicates are shown. The bar indicates 5 % sequence divergence. The phylogenetic distance of the tree between organisms is the sum of the horizontal segments.

belongs to *L. plantarum* subsp. *plantarum*.

Lactic acid production capacity of LP TO-A

The supernatant was collected from medium in which LAB was cultured for 2 days under anaerobic conditions, and the lactic acid concentration in the supernatant was measured (Figures 2A and S1). The lactic acid concentration in the culture supernatant of LP TO-A was higher than in the culture supernatant of other LAB, and the L-lactic acid concentration in the supernatant of LP TO-A was the second highest compared to other LAB (Figure 2A). Moreover, the amount of D-lactic acid produced by LP TO-A did not differ markedly from that produced by *L. plantarum* ATCC14917, but the amount of L-lactic acid produced by LP TO-A was approximately 2.88-fold higher than that produced by *L. plantarum* ATCC14917 (Figure 2A). The culture supernatant of LP TO-A contained approximately 264 mM lactic acid. By measuring the absorbance over time, we investigated the proliferation capability of LP TO-A compared with *L. plantarum* ATCC14917. Consequently, the ultimate absorbance value of LP TO-A was higher than that of *L. plantarum* ATCC14917, and LP TO-A transitioned to the logarithmic growth phase earlier than *L. plantarum* ATCC14917 (Figure 2B). However, the ultimate absorbance value of LP TO-A was lower than that of *L. rhamnosus* ATCC53103, and LP TO-A transitioned to the logarithmic growth phase later than *L. rhamnosus* ATCC53103 and *L. gasseri* ATCC19992 (Figure S2).

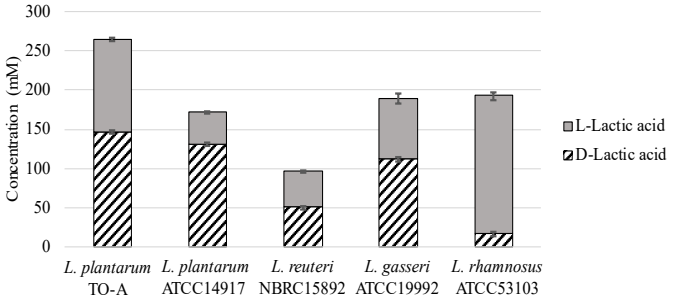

Figure 2(A): Comparison of the lactic acid production ability of various LAB. D/L-lactic acid concentrations in the LAB culture supernatant were calculated based on D-lactic acid and L-lactic acid calibration curves. Values indicated are the mean ± SEM (n = 3).

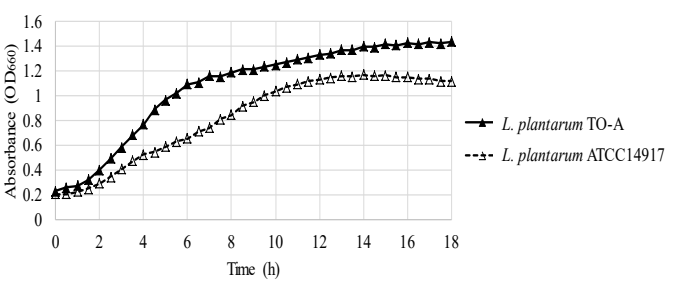

Figure 2(B): Proliferation curves of *L. plantarum* strains in MRS broth under anaerobic conditions. The absorbance (OD_{660}) was measured every 30 min for 18 h. Values indicated are the mean (n = 3).

In vitro growth inhibition activity of LP TO-A against pathogenic bacteria

To compare the growth inhibition activity of LP TO-A with other LAB, we observed the generation of inhibition circles formed by LAB under anaerobic conditions on lawns of pathogenic bacteria known as causative agents of opportunistic infections (*E. coli* ATCC8739, MRSA, *C. perfringens* ATCC13124 and *C. difficile* ATCC17859) (Figure 3). These experiments were based on results shown in Figure 2, which indicated that LP TO-A produces sufficient lactic acid to inhibit the proliferation of pathogenic bacteria. Consequently, LP TO-A formed an inhibition circle against all pathogenic bacteria tested, and the inhibition circles produced by LP TO-A were larger than those of *L. reuteri* NBRC15892 and *L. gasseri* ATCC19992. In addition, the inhibition circles formed by LP TO-A resembled the inhibition circles produced by *L. plantarum* ATCC14917 and *L. rhamnosus* ATCC53103. However, the inhibition circle could not be clearly discerned for *C. difficile* ATCC17859 because the lawn was not as thick as that on the other LAB plates. In the presence of culture supernatant of each LAB, proliferation of the pathogenic bacteria was assayed over time by monitoring the absorbance. The proliferation of each bacterial pathogen was greatly suppressed by the culture supernatant of LP TO-A (Figure 4). In particular, compared with other LAB culture supernatants, the culture supernatant of LP TO-A produced the strongest growth inhibition activity against *E. coli* ATCC8739, *C. perfringens* ATCC13124 and *C. difficile* ATCC17859 (Figures 4A, 4C,4D). Moreover, the culture supernatant of LP TO-A suppressed the proliferation of MRSA to the same degree as the culture supernatant of *L. gasseri* ATCC19992 and *L. rhamnosus* ATCC53103 (Figure 4B).

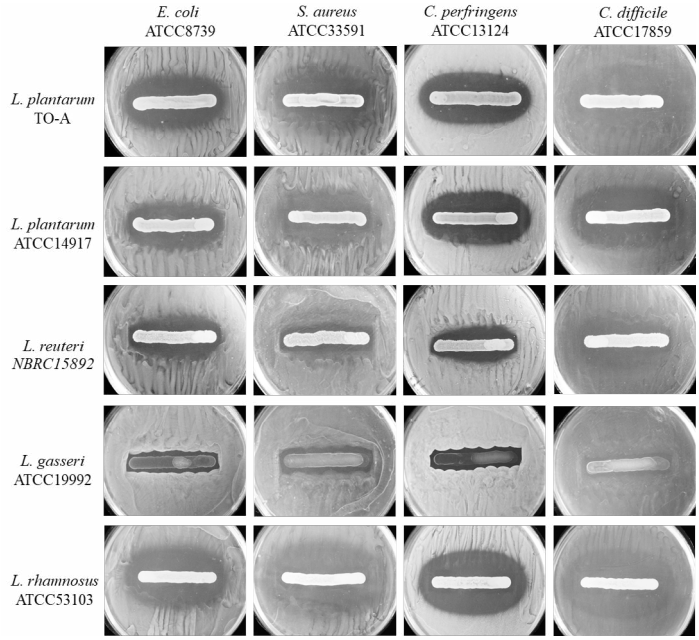

Figure 3: Zones of inhibition produced by LAB.
Circular inhibition zones formed by various LAB co-cultured various pathogenic bacteria for 2-3 days under anaerobic conditions.

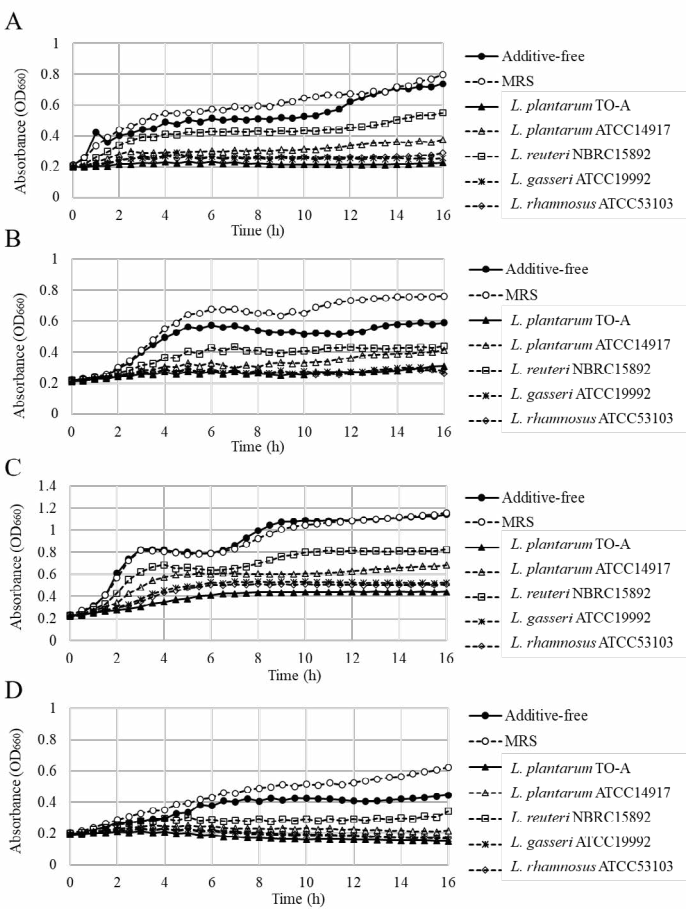

Figure 4. Growth inhibition assay using LAB culture supernatant.
E. coli ATCC8739 (A), MRSA (B), *C. perfringens* ATCC13124 (C) and *C. difficile* ATCC17859 (D) were grown in BHI broth including 1 % glucose and LAB culture supernatant under anaerobic conditions. Pathogenic bacteria cultured in BHI (including 1 % glucose) broth and BHI (including 1 % glucose and MRS) broth were used as a control. The absorbance (OD_{660}) was measured every 30 min for 16 h. Values indicated are the mean (n = 3).

LP TO-A protects host nematodes against MRSA infection

To investigate whether LP TO-A suppresses the effects of MRSA infection in host nematodes; we performed survival assays using *C. elegans* (Figure 5). Nematodes pre-fed LP TO-A exhibited significantly increased survival time following MRSA challenge compared with nematodes pre-fed *E. coli* OP50 (Figure 5B). The maximum survival time of LP TO-A fed nematodes was 120 h longer than that of *E. coli* OP50 fed nematodes (Figure 5B). These data indicate that LP TO-A suppresses the effects of MRSA infection in host nematodes.

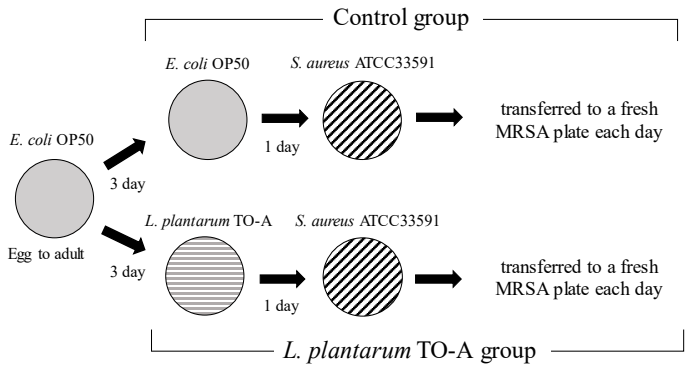

Figure 5 (A): Survival of *C. elegans* exposed to MRSA.

Schematic illustration of the survival assay. Adult nematodes were cultured on a lawn of *E. coli* OP50 or LP TO-A for 1 day, after which adult nematodes were exposed to MRSA and scored for survival each day.

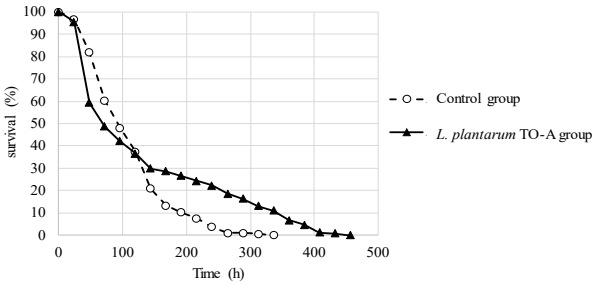

Figure5(B): The survival rate is indicated and calculated as the mean of three independent experiments (*E. coli* OP50: n = 187, LP TO-A: n = 251). ** $p < 0.01$ determined using the log-rank test that was performed using SPSS software.

LP TO-A mediated inhibition of MRSA adhesion

Figure 6A shows the percent adherence of each LAB relative to that of *L. plantarum* ATCC14917. The percent adherence of LP TO-A on collagen was lower than that of *L. plantarum* ATCC14917 but higher than that of *L. gasseri* ATCC19992 and *L. rhamnosus* ATCC53103. However, the percent adherence of LP TO-A for mucin was higher than that of *L. plantarum* ATCC14917 but lower than that of LAB other than *L. plantarum* ATCC14917. Previous reports indicate that *L. reuteri* NBRC15892 exhibits very high adherence to mucin [22,23], and our experiments showed that *L. reuteri* NBRC15892 adheres more strongly to mucin compared to other LAB (Figure 6A). These results suggest that although LP TO-A adheres to collagen and mucin, it does not do so more strongly compared with other LAB. However, the ability of LP TO-A to displace MRSA adhering to collagen and mucin was similar to that of other LAB (Figures 6B and 6C). In particular, LAB displaces MRSA adhering to mucin more strongly than MRSA adhering to collagen, and LP TO-A

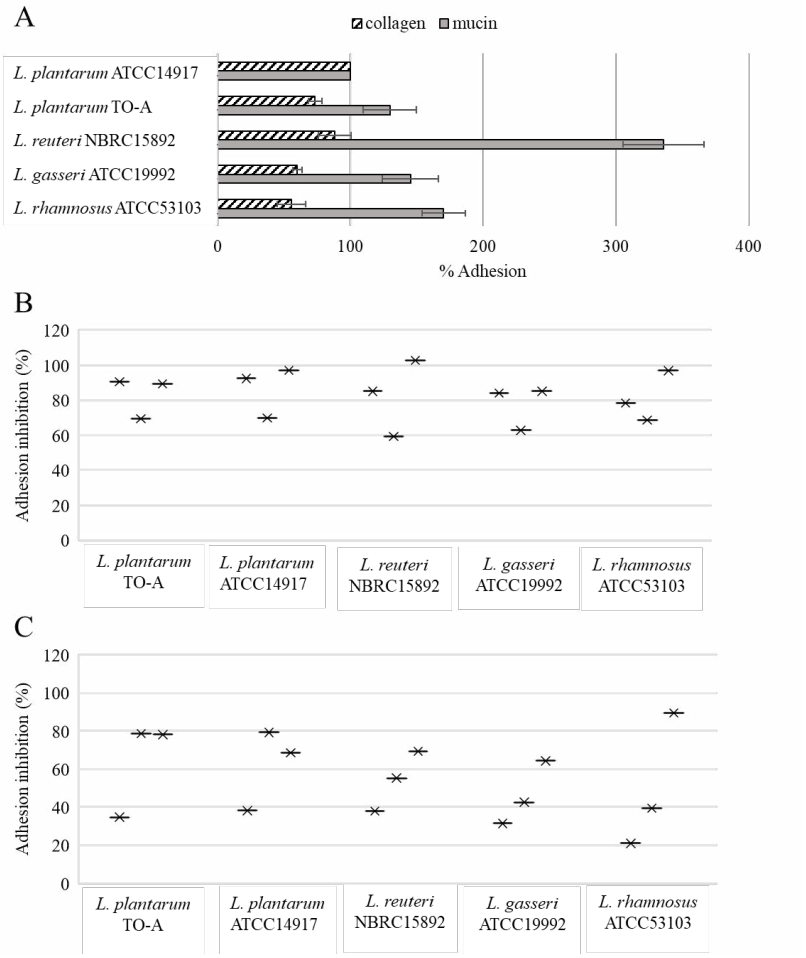

Figure 6. Inhibition of adherence of MRSA to collagen and mucin by LAB. (A) Rates of LAB adhesion to collagen and mucin are shown as relative to the rates of *L. plantarum* ATCC14917, which were defined as 100 %. Values indicated are the mean ± SEM (n = 3). The fluorescence intensity of natural MRSA on collagen and mucin was defined as 100 %, and the calculated rates of inhibition of MRSA adherence to collagen (B) and mucin (C) by each LAB are shown. Each point shows the calculated value of three independent experiments.

displaced up to 65.3 % of MRSA cells adhering to mucin (Figure 6C). No correlation was observed between the ability of LAB to adhere to the intestinal mucosa and removal of MRSA adhering to the intestinal mucosa (Figure 6).

DISCUSSION

With the significant reclassification of LAB in 2020 the scientific name of *Lactobacillus plantarum* became *Lactiplantibacillus plantarum*, which was composed of two subspecies, that is, *L. plantarum* subsp. *plantarum* and *L. plantarum* subsp. *argentoratensis*, and it was also reported that the *rec*A and *cpn*60 gene sequences

are effective for subspecies discrimination [11]. Although the sequences of both subspecies were closely related, the clusters in the MLSA phylogenetic tree of this study clearly diverged, as Li, Liu, and colleagues proposed that *L. plantarum* subsp. *argentoratensis* be considered a species [11, 24]. Our study confirmed that LP TO-A clearly belongs to the *L. plantarum* subsp. *plantarum* cluster (Figure 1), and as already shown in previously reports, the MLSA method is effective for bacterial species identification [25-30]. In addition, based on results of fermentation tests using API 50 CH, *L. plantarum* ATCC14197, LP TO-A, *L. paraplantarum* JCM12533, and *L. argentratensis* NBRC106468 were classified as "*Lactobacillus plantarum* 1", but high similarity to LP TO-A was only shown for *L. plantarum* ATCC14197 (Tables 1 and S1). Based on the above results and current classification criteria, LP TO-A was judged to belong to *L. plantarum* subsp. *plantarum*.

Furthermore, our analyses indicate that LP TO-A has higher lactic acid production capability than *L. plantarum* ATCC14917, *L. gasseri* ATCC19992, *L. reuteri* NBRC15892, and *L. rhamnosus* ATCC53103 (Figure 2). Lactic acid is known to have many beneficial effects on the host in addition to inhibiting the growth of pathogenic bacteria [31-33]. Moreover, the minimum bactericidal concentration of lactic acid for gram-negative and gram-positive pathogenic bacteria is reportedly only 28.1 mM [34]. LP TO-A can also be expected to have beneficial effects on the host similar to other LAB because our experiments showed that LP TO-A produces approximately 264 mM lactic acid (Figure 2a). In this study, we showed that the ultimate absorbance value of LP TO-A was higher than that of *L. plantarum* ATCC14917, the same as that of *L. gasseri* ATCC19992 and *L. reuteri* NBRC15892, and lower than that of *L. rhamnosus* ATCC53103 (Figures 2b and S2). These results suggest that the high lactic acid concentration in the culture supernatant of LP TO-A is not associated with the proliferative capacity of LP TO-A and indicate that LP TO-A can produce very high levels of lactic acid.

The growth inhibition activity of probiotic bacteria is known to depend on culture conditions. However, LP TO-A exhibited growth inhibition activity against pathogenic bacteria in two experiments with different culture conditions (Figures 3 and 4). In addition, we revealed that LP TO-A inhibits the growth of both gram-positive and gram-negative pathogenic bacteria. These results show that LP TO-A exhibits growth inhibition activity similar to that of other LAB and suggest that the growth inhibition activity of LP TO-A against various pathogenic bacteria does not depend on culture conditions.

We also revealed that LP TO-A prolongs the survival time of nematodes in the presence of MRSA (Figure 5). Some *L. plantarum* strains are known to regulate the *C. elegans* immune system in addition to inhibiting the growth of pathogenic bacteria [35,36]. Therefore, LP TO-A is thought to both inhibit the growth of pathogenic bacteria and regulate host immunity to suppress the effects of bacterial infection, similar to other *L. plantarum* strains. Although the *C. elegans* intestine is a simple structure composed of only 20 cells, the *C. elegans* intestinal epithelial cells are very similar to those of mammals. The *C. elegans* glycocalyx, which is composed of a layer of glycoproteins such as mucin, is formed just on the outside of membranous microvilli and serves as a physical barrier to bacteria [37]. We hypothesize that the preventative effect of LP TO-A on pathogenic bacterial infection in the *C. elegans* intestine may also occur in the human gut.

Our *in vitro* experiments indicated that LP TO-A suppresses the adhesion of MRSA onto the intestinal mucosa (Figure 6). The LAB used in probiotic preparations must be able to act on pathogenic bacteria that adhere to the intestinal mucosa, because some pathogenic bacteria that adhere to the intestinal mucosal layer are known to damage the mucosal barrier and epithelial cells [38]. The data shown in Figure 6 suggest that LP TO-A meets the above criterion. We did not find a correlation between the ability of LAB to adhere to the intestinal mucosa and the ability of LAB to displace MRSA adhering to the intestinal mucosa. This result suggests that an as yet unknown function of LAB may be involved in the removal of the MRSA adhering to the intestinal mucosa. We speculate that this unknown function of LAB involves the production of a bacteriocin and that the bacteriocin produced by LP TO-A displaces MRSA adhering to the intestinal mucosa of the host. Many *L. plantarum* strains are known to produce bacteriocins, and the extract of bacteriocin produced *L. plantarum* reportedly inhibited the adhesion of *S. aureus* to the intestinal mucosa [9,39,40].

The annual incidence of bacterial infections is increasing worldwide, and one cause is the existence of drug-resistant bacteria [41,42]. Since the emergence of penicillin-resistant *S. aureus* in the 1950s to this day, the number of strains that have acquired drug resistance has increased every year [43]. The abuse and misuse of antibiotics exerts a continuous selection pressure on bacteria that has resulted in the emergence and expansion of the distribution of various drug-resistant bacteria. The One Health concept was introduced in recent years and has been promoted as a means to overcome this situation [44]. The One Health approach is an effort to maximize the health of humans, animals, and the environment. This approach includes eliminating the improper use of antibiotics and introducing measures to maintain the efficacy of existing antibiotics [45]. In this study, we found that LP TO-A inhibits the growth of various pathogenic bacteria —including drug-resistant bacteria— both *in vitro* and *in vivo*. The results of our study suggest that LP TO-A is useful alternative to antibiotics, and we believe that LP TO-A can prevent and treat infectious disease caused by drug-resistance bacteria such as MRSA.

AUTHOR CONTRIBUTIONS

Investigation, R.S.; writing—original draft preparation, R.S and N.S.; project administration, N.S.; supervision, N.S. All authors have read and agreed to the published version of the manuscript.

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CONFLICTS OF INTEREST

The authors declare they have no conflicts of interest.

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