Role of Plantaricin Produced by Lactobacillus plantarum on Fermentation of Ishizuchi-kurocha and its Application in Post-fermented of Coarse Tea (Bancha) using the Artificially Fermented Method

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(石鎚黒茶の発酵における *Lactobacillus plantarum* 生産プランタリシンの役割と人工発酵法を用いた番茶の後発酵への応用について)

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Contents

1. OVERVIEW

Lactobacillus plantarum is a lactic acid bacterium found in nutrient-rich environments such as plants, meat, fish, and dairy products. *L. plantarum* produces organic acids, fatty acids, ammonia, hydrogen peroxide, diacetyl, and bacteriocin as well as other substances to grow and survive in its environment [1]. The bacteriocin produced by *L. plantarum* is known as plantaricin and is generally reported as a class II bacteriocin, a very broad class with a variety of bactericidal/bacteriostatic mechanisms [2]. Class II bacteriocins are small peptide (< 10 kDa), heat-stable molecules with isoelectric points varying from 8.3 to 10.0 [3]. They have an amphiphilic helical structure that allows for their insertion into the cytoplasmic membrane of the target cell, thereby promoting membrane depolarization and cell death [4].

The uses of plantaricin are widely studied and has developed rapidly, with diverse applications such as antibacterial packaging film [5], reduction of intestinal cancer cells [6], bio-preservation of fresh fish and shellfish [7], extending the shelf-life of food without altering the nutritional quality of products [8], anti-cancer drugs [9], and active polyvinylidene chloride (PVDC) films as antimicrobial wrapping for fresh pork [10]. Plantaricin products can lower cholesterol, act as antioxidants [11], and are active against *Candida* [12]. However, most reports concentrate on plantaricin as a bio-preservative, owing to its use in the extension of shelf life and effectiveness against a range of harmful bacteria.

Plantaricin from *L. plantarum* has been widely reported, and the broad, heterologous nature of plantaricin has been emphasized by several authors. Differences in structural amino acids result in different characteristics of plantaricins, such as resistance to pH and temperature [13], and antimicrobial activity [14]. Differences are also influenced by the location of the plantaricin-coding genes [3]. In *L. plantarum,* these are located in operon clusters, which may be located on chromosomes, plasmids, or transposons [15][3]. The mechanism used to inhibit and kill pathogenic bacteria depends on the characteristics of the plantaricin; different classes

have different mechanisms. Commonly, plantaricin disrupts cell wall integrity and inhibits protein or nucleic acid synthesis [2]. It has been reported that the bacterial membrane is the target of bacteriocins [16]; therefore, it is worth summarizing and clarifying the mechanisms underlying the bactericidal/bacteriostatic activity of plantaricin against pathogenic bacteria.

In this research, we present information about the diversity, characterization, and heterologous expression of plantaricin from *L. plantarum* that has many benefits to human life, with the aim of promoting its use and stimulating innovation in a diverse range of industries, especially the food industry for food preservation.

2. EXPERIMENT

2.1 Plantaricin A of *Lactobacillus plantarum* IYP1718 Plays a Role in Controlling Undesirable Organisms in Soil

2.1.1 Introduction

Lactic acid bacteria (LAB) in soil have roles such as enhancing decomposition, release of plant nutrition, and increasing soil humus formation by altering the organic materials. During decomposition of organic materials in soil, gas and heat are produced, resulting in loss of energy to a cultivated crop, causing harm to plants. LAB facilitate decomposition of organic matter, resulting in less energy loss to excess heat and gas [17]. Further, *Lactobacillus spp.* can help neutralize soil and remove byproducts that can form a harmful environment. The presence of *Lactobacillus* can inhibit the undesirable organisms in soil to form a balanced environment that can support plant life. *Lactobacillus* thus contributes to decomposition and disease suppression[18].

Black rot is one of the diseases affecting Brassica plants and is caused by *Xanthomonas campestris pv. Campestris* [19]. *X. campestris pv. campestris* can spread quickly to other brassica plants when water splashes from one plant to another. The symptoms of black rot disease are yellow, wedge-shaped patches on the leaf edges [19]. *Lactobacillus* can control the growth of fungi, yeast, and aerobic bacteria [18]. One of the *Lactobacillus spp.* that can control undesirable organisms is *Lactobacillus plantarum. L. plantarum* can control the growth of undesirable organisms by express secondary metabolites such as bacteriocin [20]. In this study, *L. plantarum* was isolated from healthy soil and soil that has black rot disease in brassica plants, to compare the expression of the bacteriocin, plantaricin A. Plantaricin is a peptide that usually has membrane-permeabilizing activity and contains between 25-60 residues amino acid residues. *plnA* has antimicrobial activity and depends on a nonchiral interaction with lipids and the target cell membrane [21].

plnA expressed by *L. plantarum* inserts in the cytoplasmic membrane of the target cell, thereby promoting membrane depolarization and cell death [2]. This research aims to determine the existence of the bacteriocin biosynthetic cluster of *plnA* in *L. plantarum* and show the related structural cluster gene ORFs. Based on this study, *plnA* is expected to control the undesirable microorganisms in soil.

2.1.2 Materials and Methods

2.1.2.1 Isolation and Identification of *Lactobacillus plantarum*

Soil from different conditions were collected in Gifu, Japan. Collected soil included healthy soil and soil that has black rot disease in brassica plants. LAB were isolated from each sample serially diluted technique until 10^{-7} in Man, Rogosa, and Sharpe (MRS) broth and were plated on MRS agar at 37°C for 24 hours in an aerobic condition[22]. LAB were grown on MRS broth and MRS agar (Becton, Dickinson and Company - USA) and incubated at 37°C for 24 h. LAB were then selected and kept at -80°C in MRS broth with 20% glycerol.

2.1.2.2 Genomic Identification of Microorganism

2.1.2.2.1 DNA Isolation

LAB were grown in MRS Broth at 37˚C for 18 hours. Cells were harvested, and LAB genomic DNA was extracted using the Extrap Soil DNA Kit Plus Ver.2. (Nintetsu Sumikin Kankyo Kabushiki Gaisya, Japan) according to the manufacturer's protocol. Electrophoresis was performed on 1% agarose gel in Tris Acetic acid EDTA (TAE 1X) buffer and photographed under UV light.

2.1.2.2.2 16S rRNA Sequencing

The 16S rRNA gene fragment of ~1.5 kb was amplified using a pair of universal primers 27 F: (5′- GAGTTTGATCCTGGCTAG-3′) and 1525 R: (5′-AGAAAGGAGGTGATCCAGCC-3′) [22]. Polymerase chain reaction (PCR) was carried out in a Fast reaction Tube (Applied Biosystems, USA) in a total volume of 25 µl containing 12.5 µl 2 × Green Master Mix PCR (Promega, USA), 1.25 µL of each primer 27F and 1492R (concentration 0.05 pmol/µL), 9 µL nuclease free deionized water, and 1 µL template DNA, and was run under the following temperature program: initial denaturation of DNA for 5 min at 95°C, 25 cycles of 1 min at 94°C, 1 min at 56°C, and 1.5 min at 72°C; and final extension for 7 min at 72°C. Then, 5 µl aliquots of the PCR product were analyzed by electrophoresis using 1% (w/v) agarose gel in TAE 1X

buffer at 100 V for 30 min. The gel was then placed in an Electronic U.V. Transilluminator to detect the presence a 1500 bp band. The size of the DNA fragments was estimated using a FastGene 100 bp DNA Ladder (Nippon Genetics, Germany). Fast Gene™ Gel/PCR Extraction kit (Nippon Genetics, Germany) was used for purification before sending the extracted DNA for sequencing, according to the manufacturer's instructions. An average of 500 bp nucleotides for each sequence from each side was read and compared against the NCBI database using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Six isolates had been chosen from healthy soil and four isolates were chosen from soil that has black rot disease in brassica plants. Isolates with 98% or higher similarity in sequences were identified as the same species.

2.1.2.2.3 Phylogenetic Tree Construction

The DNA sequences obtained were then assembled into contiguous sequences (contigs) using DNAStar; about over a hundred genomic contigs were edited using BioEdit and were aligned using the Bioedit-ClustalW Multiple Alignment. A phylogenetic tree was drawn using Mega7 Construct/ Test Neighbor-Join Tree with 1000 bootstrap replicates.

2.1.2.3 Plasmid Extraction from *L. plantarum* isolated from Soil

Plasmids were extracted with a protocol based on *Lactobacillus spp.*-oriented High-Quality methods[23] with some modification. *L. plantarum* was cultured in 2 ml for 18 h. The culture was centrifuged at 15000 rpm for 5 min. The supernatant was removed, and the pellet was resuspended in 25% sucrose containing 30 mg/mL lysozyme, with a final volume of 200 µL. Next, 200 µL of 0.5M EDTA was added to the solution and incubated at 37°C for 15 min. The solution was mixed with 400 µL of alkaline SDS solution (3% SDS on 0.2N NaOH), incubated for 7 min at room temperature, and then added with 300 µL of ice-cold 3M sodium acetate (pH 4.8). The solution was mixed immediately and centrifuged at 14000 rpm for 15 min at 4°C. Supernatant was transferred to a new tube and added with 650 µL of isopropanol (R/T), mixed immediately, centrifuged at the max speed for 15 min at 4°C. All the liquid was removed and

the pellet was resuspended in 320 µL of sdH_2O, 200 µL of 0.5M EDTA, 200 µL of 7.5M ammonium acetate, and 350 µL phenol: chloroform (1:1). The solution was mixed immediately and centrifuged at the max speed for 5 min at room temperature. The upper phase was transferred to a new tube and added with 1 ml ethanol. The solution was mixed immediately and centrifuged at the max speed for 15 min at 4°C. The supernatant was discarded and the pellet was resuspended in 40 µL of TE buffer.

2.1.2.4 Identification of the Plantaricin Gene from *L. plantarum* Using Specific Primers

Identification of plantaricin was performed using PCR. Specific primers of the bacteriocin used in this study are shown in Table 1. The amplicons were purified using a purification kit (Nippon Genetics, Germany) and DNA Sequence was determined using a Multi – capillary DNA Sequencer "ABI Prism 3100/3130 Genetic Analyzer" (Gifu University, Japan).

Table 1 Primers used throughout this study and their amplification details

Name	Sequence (5′ → 3′)	Annealing	Reference
plnA-F	TAGAAATAATTCCTCCGTACTTC	55	[1]
plnA-R	ATTAGCGATGTAGTGTCATCCA		
plnEF-F	TATGAATTGAAAGGGTCCGT	54	[1]
plnEF-R	GTTCCAAATAACATCATACAAGG		
plnW_F	CACGTCACAGCTAATCTGG	61.5	[24]
plnW_R	CTAATTGCTGAATGGTTGGT		
plnS_F	GCCTTACCAGCGTAATGCCC	56	[25]
plnS_R	CTGGTGATGCAATCGTTAGTTT		
plnNJK_F	CTAATAGCTGTTATTTTTAACC	55	[26]
plnNJK_R	TTATAATCCCTTGAACCACC		
pln423A_F	GTCGCCCGGGAAATACTATGGTAATGGGG	58	[27]
pln423A_R	GCGTCCCGGGTTAATTAGCACTTTCCATG		

2.1.2.5 DNA Sequencing

Plasmid DNA Sequencing was performed using the Multi – capillary DNA Sequencer "ABI Prism 3100/3130 Genetic Analyzer" (Gifu University, Japan). Sequences were translated to amino acids using the CLC Sequence Viewer program. Computer alignment and BLAST (basic local alignment search tool) analysis of the sequence were performed using BioEdit for Windows.

2.1.3 Results

2.1.3.1 Isolation and Identification of *L. plantarum* from Soil

Six LAB isolates from healthy soil and four LAB isolates from soil that has black rot disease in brassica plants were isolated. Their morphology on the MRS agar plate presented as milky-white in color, circular and convex. The 16SrRNA locus for LAB was amplified using universal primers to confirm the species. The amplicon products of 1500 bp size were used for species identification. All the sequences were found to be *L. plantarum.* The potential *L. plantarum* from soil that has black rot disease in brassica plant was chosen for the next experiment. *L. plantarum* isolated from soil that has black rot disease in brassica plant was designated as *L. plantarum* IYP1718. The sequence of *L. plantarum* IYP1718 was deposited to NCBI Data GenBank under the accession number MK743941.

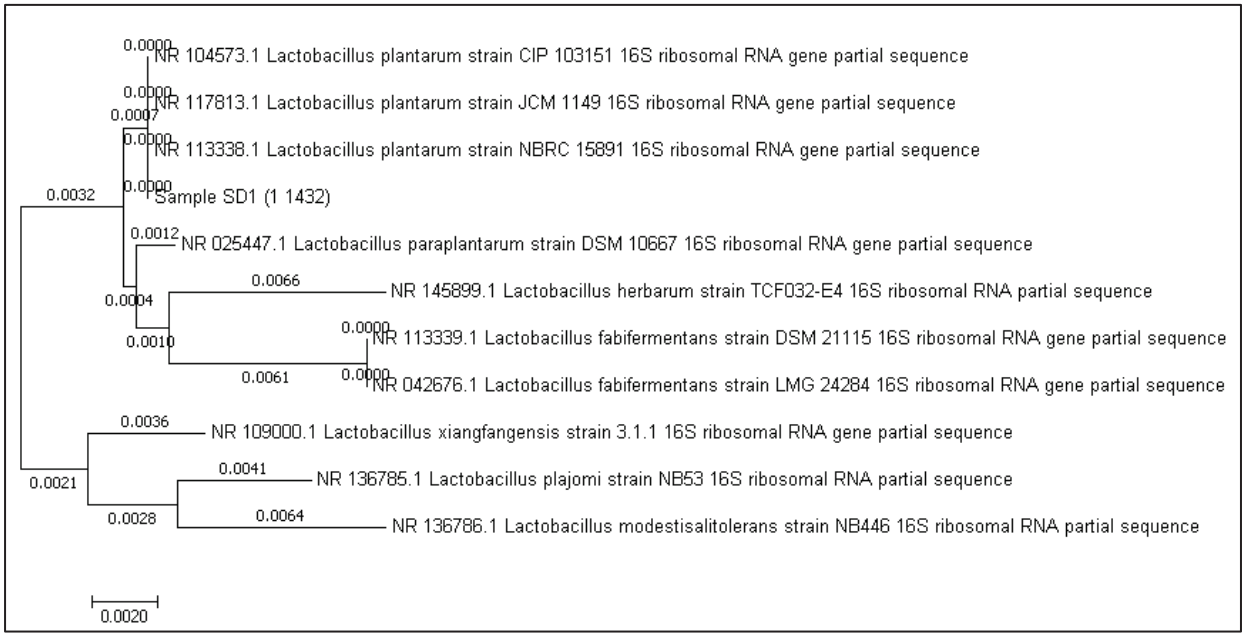

Fig. 1 Phylogenetic tree of *L. plantarum* based on the 16S rRNA sequence analyses using Mega7 Construct/Test Neighbor-Join Tree, showing the phylogenetic placement of representative strains; Sample SD1 is *L. plantarum* IYP1718

A phylogenetic tree was drawn to determine the closeness of the relationship of the species based on their genetic similarities and differences (Fig. 1). A phylogenetic tree of *L.*

plantarum based on 16S rRNA sequence analyses was constructed using Mega7 Construct/Test Neighbor-Joining Tree, which showed the phylogenetic placement of representative strains. The result obtained from the 16S rRNA sequences revealed 100% similarity with the 16S rRNA sequence of *L. plantarum.*

2.1.3.2 Identification of Plantaricin Gene from *L. plantarum* Using Specific Primers

Six specific primers of plantaricin were used for amplification, but only *plnA* was found to exist in *L. plantarum*; the other plantaricins could not be detected by PCR. Cells were harvested at the beginning of the stationary phase to reach the maximum production of *plnA* (data not shown). The genes encoding bacteriocins are located on operon clusters, which may be placed on plasmids. Plasmid isolation showed that all *L. plantarum* isolates had plasmids. All plasmid of *L. plantarum* were approximately ~10 kbp. The results showed that a fragment DNA containing the *plnA* gene of approximately ~550 bp was amplified from the plasmid of *L. plantarum.* The presence of the *plnA* gene encoded on the plasmid was thus confirmed (Fig. 2) based on the observation of the PCR product using specific primers for *plnA*. Among the six isolates of *L. plantarum* from healthy soil, three produced *plnA*, whereas one of four *L. plantarum* isolates from soil that has black rot disease in brassica plant produced *plnA* (Fig. 2). *L. plantarum* COY2906 isolated from Virgin Coconut Oil that had confirmed presence of *plnA* was used as a positive control.

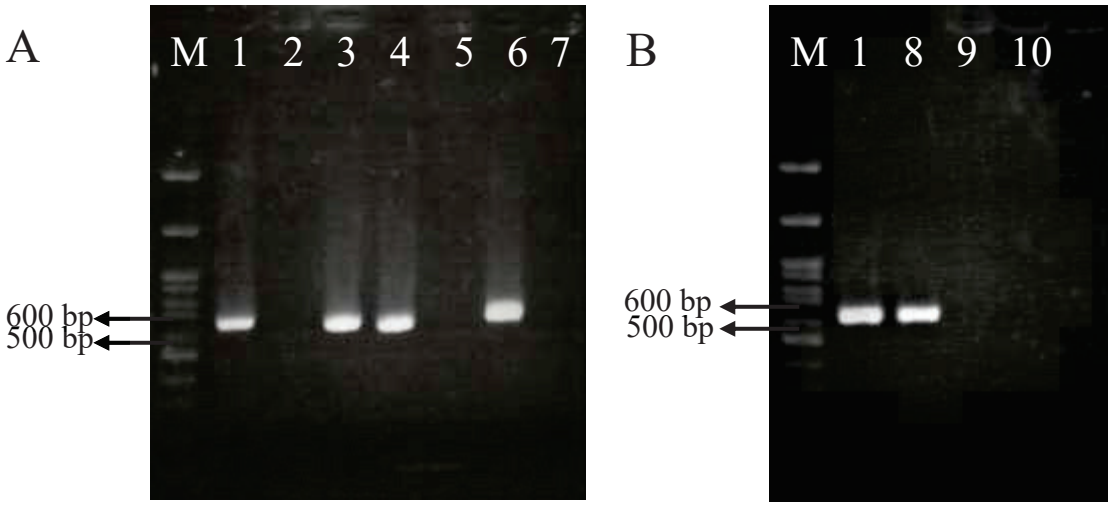

Fig. 2 A. Detection of *plnA* sequence in *L. plantarum* plasmid isolated from healthy soil **B.** Detection of *plnA* sequence in *L. plantarum* plasmid isolated from soil with black rot disease in brassica plants. M**:** Molecular Weight Marker 100bp DNA Ladder**.** 1. Positive control, Amplification of *plnA* from *L. plantarum* COY2906 isolated from Virgin Coconut Oil; **2-10.** Amplification of *plnA* from *L. plantarum*

2.1.3.3 Sequence Analysis

The potential *L. plantarum* IYP1718 from soil that has black rot disease in brassica plants was chosen for sequence analysis. The identity of the amplified *plnA* gene was confirmed by DNA sequence analysis, whereby high DNA sequence identity confirmed 100% correspondence to *plnA* from GenBank originating from *L. plantarum* with accession number AFJ79564.1. This sequence of ~528 bp was translated using CLC Sequence Viewer. The DNA sequence of *plnA* was analyzed to determine the start – end position of transcription and translation. Computer analysis of the ORF indicated *plnA* translation start at the first AUG codon. The DNA sequence from the sample using a specific primer showed that the start position of transcription and translation is 60 and that the end position of transcription and translation is 206.

Bioedit ClustalW Multiple Alignment was used for sequence alignment. *plnA* from GenBank was compared with the *plnA* from *L. plantarum* IYP1718. *No differences were observed between plnA* GenBank and *plnA* from *L. plantarum* IYP1718, and both sequences

were identical with *100% similarity homology between plnA* from GenBank and *plnA* from *L. plantarum* IYP1718. Structurally, *plnA* has an Open Reading Frame (ORF) of 147 bp that encoded 48 amino acid residues (Fig. 4). Comparison of the amino acid sequence to known proteins in the database revealed significant homology with class II bacteriocins.

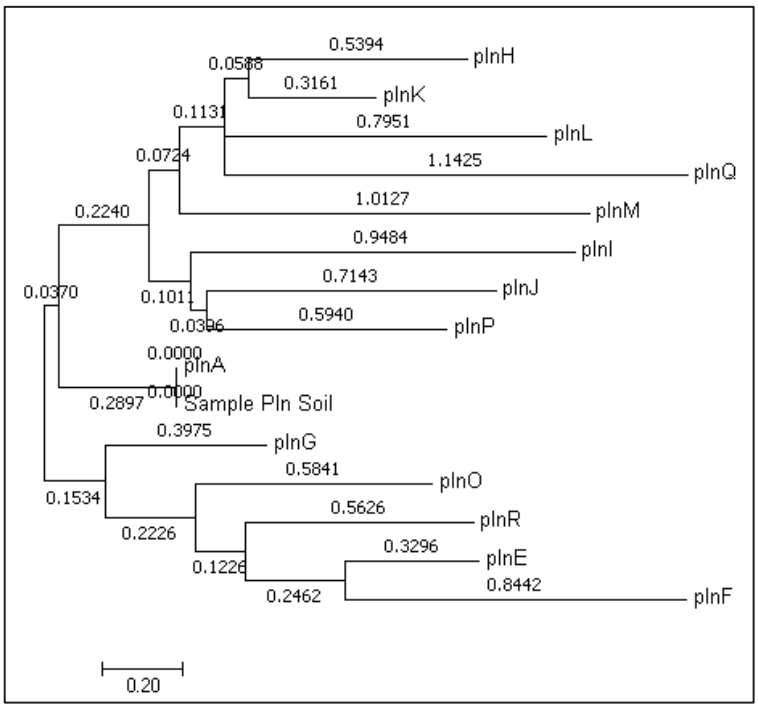

Fig. 3 Phylogenetic tree based on the sequence analyses of *plnA* between *L. plantarum* IYP1718 and plantaricin from *L. plantarum* GenBank. The phylogenetic tree shows the closeness of the relationship of plantaricin based on the genetic similarities.

It was found that *plnA* from *L. plantarum* IYP1718 and GenBank showed 18% *similarity* with Plantaricin G based on pairwise distance (Fig. 3) (data not shown).

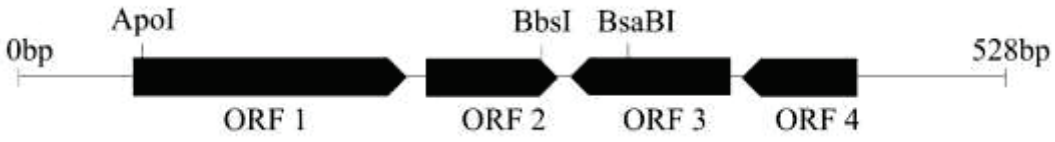

Fig. 4 Schematic representation of the *plnA* gene cluster. ORF 1 indicates the structural gene *plnA*.

Even though *L. plantarum* IYP1718 DNA sequence analysis showed high DNA sequence identity of 100% corresponding to *plnA* from GenBank, the plantaricin gene was observed elsewhere. The cluster structure of the plantaricin encoding region was not shown around the *plnA* gene region. The DNA fragment containing the *plnA* operon as the probe revealed extensive homology, has several restriction enzyme sites: *ApoI, BbsI, and BsaBI* (Fig. 4). The first ORF encodes a protein consisting of 48 amino acid residues, followed by the TAA stop codon. Computer analysis of the ORF indicated that the *plnA* translation starts at the first AUG codon. ORF1 was identified as *plnA* that consist of 146 bp, ORF 2 was identified as Structure of Importin Beta Bound to the Ibb Domain of Importin Alpha that consists of 71 bp. However, the function of ORF2 has not been related to any bacteriocin production. Computer analysis showed ORF3 and ORF4, but these could not be identified in NCBI GenBank and encoded for a peptide with unknown function. All ORFs larger than 20 bp were compared against the protein database using the BLAST server as shown in Table 2.

Table 2. Characteristics of the predicted ORFs encoded from *L. plantarum* IYP1718

ORF	Location (bp)	Size (bp)	Gene	Protein accession no.	% Identity
ORF1	60-206	146	*plnA*, *L. plantarum*	AFJ79564.1	100%
ORF2	230-311	71	Structure of Importin Beta Bound to the Ibb Domain of Importin Alpha	1QGK_A	42%
ORF3	342-428	86	undetected		
ORF4	444-506	62	undetected		

Multiple-sequence alignment was performed based on bacteriocin class IIa to show closeness relative to other bacteriocins. Pairwise distance confirmed that the amino acid

sequence of *plnA* showed 22% similarity with Enterococcin from *Enterococcus faecalis* BFE 1071 based on the pairwise distance (data not shown). It remains to be determined whether *plnA* has the promoter motifs of the plantaricin operon with a similarity of 22% with enterococcin (Fig. 5).

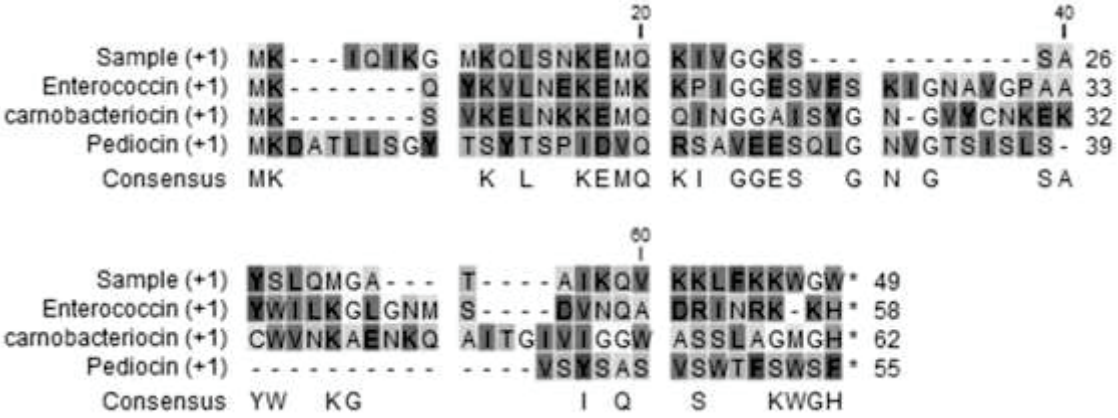

Fig. 5 Multiple-sequence alignment of known and putative bacteriocin precursors using Bioedit ClustalW Multiple Alignment. The known sequences are *Enterococcin, Carnobacteriocin BM1*, and Pediocin

2.1.4 Discussion

In this study, *L. plantarum* was isolated *from* healthy soil and soil with black rot disease in brassica plants. Six species of *L. plantarum* isolated from healthy soil showed three *L. plantarum* isolates with *plnA*, whereas one of four *L. plantarum* isolates from soil with black rot disease in brassica plants had *plnA*. *plnA* from *L. plantarum* of healthy soil plays a role to control the undesirable organisms in soil in order to form a balanced environment that can support plant life. Under healthy conditions, *L. plantarum* produced more plantaricin compared to that in *L. plantarum* isolated from soil with black rot disease in brassica plant. *L. plantarum* that did not have *plnA* indicated an incompatible plasmid. The plasmids that could not receive this gene are considered to be deficient in some genes and specific function that allow compatible plasmids in the cell to accept plantaricin genes. Moreover, if the plasmid is incompatible, it is released from the cell. The manner of transfer depends on their mechanism of replication in a single cell. This mechanism helps control the undesirable organisms in soil to form a balanced environment that can support plant life.

L. plantarum IYP1718 isolated from soil with black rot disease in brassica was selected for the next experiment because of its ability to survive in soil that has black rot disease. It was confirmed that the *plnA* biosynthetic cluster was located on a plasmid of *L. plantarum*. *L. plantarum* IYP1718 survived in an environment that has an undesirable organism by producing *plnA*. This is obtained through *L. plantarum* from healthy soil and transfer of genetic material from one bacterial cell to *L. plantarum* IYP1718, either through direct contact or a bridge between the two cells, but some plasmids contain genes called transfer genes that facilitate the beginning of conjugation.

During their life cycle, bacteria must adapt to several environments, by coping with environments containing few nutrients and with other bacteria that produce the required metabolic products. *plnA* is a secondary metabolite that also function as self-defense from

undesirable organisms. *plnA* can dissipate the proton motive force by disrupting the trans-membrane potential of the sensitive cell. Two bacteriocin peptides appear to form relatively specific pores, thus dissipating the trans-membrane potential[28][3].

All plasmids of *L. plantarum* were harvested in the early stationary phase to reach the maximum production. Similar to *L. plantarum* J-51, the plantaricin was detected at the end of the exponential phase and during the early stationary growth phase[29]. The 528 bp amplicon was obtained by PCR analysis of *L. plantarum* IYP1718, using a specific primer pair for the *plnA* gene. Computer analysis showed that *plnA* of the sample has an Open Reading Frame (ORF) of 147 bp that encodes 48 amino acid residues. The DNA fragment was sequenced and confirmed to have 100% identity with *plnA* from NCBI gene bank, as expected from strains of the same species. However, some reports have shown a mutation, Gly7 mutated to Ser7, in pln A of *L. plantarum* J-51. The mutation (Ser7) was located at a double-glycine leader peptide, and the putative active peptides of strain J-51 remain identical to those *plnA* peptides of *L. plantarum* C11[29]. In addition, the *plnA* encoded 47-48 amino acid residues.

plnA with 37-residues and a C-terminal moiety corresponding to the amino acid of plantaricin 423 with 19 or 18 residues has N-terminal extensions with a glycine-glycine (GG) cleavage site[27]. It is *suggested* that *plnA* from *L. plantarum* IYP1718 and GenBank showed 18% similarity with *plnG* based on pairwise distance. The 18% similarity with *plnG* indicates the closeness relative plantaricin between *plnA* and *plnG*. These peptides of class II primary and three-dimensional bacteriocin consist of two functional domains; a well-conserved hydrophilic N-terminal β-sheet domain and diverse hydrophobic or amphiphilic C-terminal α-helical domain [27].

Schematic representation showed 4 ORFs; ORF1 and ORF2 are *plnA* from *L. plantarum* and Structure of Importin Beta Bound to the Ibb Domain of Importin Alpha, respectively. ORF3 and ORF4 have undetected functions. In addition, some reports have shown that *L. brevis*

encodes brevicin 925A. Plasmid pLB925A04 carried many ORFs other than the bacteriocin-biosynthesizing gene cluster, and most of the ORFs cannot be annotated [30]. However, an ORF could describe the unknown function encoded in the *plnA* locus.

Multiple-sequence alignment was done to show the closeness relative with other bacteriocins. The amino acid sequence of *plnA* showed 22% similarity with enterococcin from *Enterococcus faecalis* BFE 1071 and 18% similarity with *plnG* based on pairwise distances. *L. plantarum* I-UL4 showed that the promoter motif of pln operon was also found in other bacteriocin systems such as the gene cluster of *sakacin A, sakacin P, carnobacteriocin A, carnobacteriocin B2,* and enterococcin A, indicating a similar regulatory mechanism for bacteriocin production [31]. That report also showed similarity with this report.

This sequence has not been fully characterized, and many genes essential for bacteriocin export, including the mode of action that regulates the production and synergistic actions of the bacteriocin are a topic of ongoing research. Class II bacteriocin production is organized within operon clusters and consists of a structural gene encoding the prepeptide, immunity gene, an ABC transporter gene, and a gene encoding an accessory protein; in some cases, presence of a regulatory gene has been reported.

2.1.5 Conclusion

L. plantarum was isolated from healthy soil and soil with black rot disease in brassica plants. Six species of *L. plantarum* isolated from healthy soil showed three *L. plantarum* isolates with *plnA*, whereas one of four *L. plantarum* isolates from soil with black rot disease in brassica plants had *plnA*. Sequenced DNA fragment confirmed to have 100% identity with *plnA* from NCBI gene bank. The amino acid sequence of *plnA* showed 22% similarity with enterococcin from *Enterococcus faecalis* BFE 1071 and *18%* similarity with *plnG* based on pairwise distances. More studies to determine the full operon and whether other genes encoding other bacteriocins are present in the *L. plantarum* IYP1718 plasmid and chromosome are required. The initiation sequence reported here will be useful for functional analysis of ORFs located in the plasmid of the *plnA* biosynthetic gene cluster in *L. plantarum*.

2.2 Effect of pH and Salinity on Lactic Acid Production and Multiplication of Plantaricin Plasmid Genes of *Lactobacillus plantarum* COY 2906 Isolated from Virgin Coconut Oil

2.2.1 Introduction

Virgin coconut oil (VCO) is a traditional essential oil, extracted from coconut milk in West Sumatra. VCO is used for making various products, such as toothpaste [32], soap [33], food products and supplements [34], cosmetics [35], and other industrial products [36], by treatments that include high or low acidity, high salt content, and/or strong heating. There are 3 ways to make VCO; heating, enzymatic processes, and fermentation. The best production process is by fermentation with lactic acid bacteria (LAB) because the process does not require heating or the addition of harmful compounds. Moreover, this allows the manufacture of a higher quality of oil than other processes. A number of bacterial species: *Lactobacillus plantarum, Corynebacterium bovis, Corynebacterium xerosis, Micrococcus luteus,* and *Lactobacillus thermobacterium* have all been reported in fermented coconut milk [37].

During fermentation, LAB produces lactic acid, acetic acid, ethanol, hydrogen peroxide and bacteriocins, each with antimicrobial activity [38], which makes the fermentation process safer. However, when converting fermented VCO into various products, it is subjected to processes which involve changes in acidity and salinity [39]. These environmental stresses will cause changes to the components involved in the growth process of LAB. In this research, we want to confirm whether LAB present in VCO could be a bio-preservative in the product, even under conditions of stress, focusing on lactic acid and bacteriocin. Changes in lactic acid will affect flavor and other characteristics, and bacteriocin is another, lesser known, anti-bacterial component, with both factors significantly affecting the product quality.

Bacteriocins production systems are genetically organized in operons, usually comprising the structural gene and genes encoding the proteins responsible for post-

translational modification and export [15][27]. *L. plantarum* produces a variety of substances with antimicrobial activity, including antimicrobial peptides collectively known as plantaricin which is encoded in plasmid and chromosome. We also investigated the correlation between lactic acid production, bacteriocin, and multiplication of plantaricin genes in *L. plantarum* COY 2906 plasmids under various stressors such as acidity and salinity.

2.2.2 Materials and Methods

2.2.2.1 Isolation and Identification of Lactic Acid Bacteria

VCO was obtained from Padang, West Sumatra, Indonesia. Isolation of LAB was carried out by serial dilution to 10^{-7} in Man, Rogosa, and Sharpe (MRS) broth. They were plated on MRS agar at 37 °C for 24 h, in anaerobic conditions [40]. Four colonies were selected randomly, based on their shape, size, and color. LAB were grown on MRS broth and MRS agar (Becton, Dickinson and Company, USA) and incubated at 37 °C for 24 h. Bacteria were then selected and kept at -80 °C in MRS broth with 20 % glycerol (Wako Pure Chemical Corporation, Japan).

2.2.2.2 Genomic Identification of Microorganisms

2.2.2.2.1 DNA Isolation from Lactic Acid Bacteria

Total bacterial DNA was extracted from LAB using the Extrap Soil DNA Kit Plus, ver. 2 (Nippon Steel & Sumikin Eco-Tech Corporation, Japan) according to the manufacturer's instructions. The quality of the extracted DNA was assessed by gel electrophoresis using a 1 % agarose gel in 1× TAE buffer, and the gel was photographed under UV light.

2.2.2.2.2 *16S rRNA* Sequencing

A 16S rRNA gene fragment (~1.5 kb) was amplified using a pair of universal primers 27 F: (5′-GAGTTTGATCCTGGCTAG-3′) and 1525 R: (5′-AGAAAGGAGGTGATCCAGCC-3′) [22]. The polymerase chain reaction (PCR) was carried out in a fast reaction tube, in a total volume of 25 µL containing 12.5 µL 2 × Green Master Mix PCR (Promega, USA), 1.25 µL of the primers 27F and 1492R (concentration 0.05 pmol/µL), 9 µL nuclease-free deionized water, and 1 µL of template DNA. This was run under the following temperature program: initial denaturation of DNA for 5 min at 95 °C, 25 cycles of 1 min at 94 °C, 1 min at 56 °C, and 1.5 min at 72 °C; and final extension for 7 min at 72 °C. Then, 5 µl aliquots of the PCR product were analyzed by electrophoresis using 1 % (w/v) agarose gel

in TAE buffer at 100 V for 30 min. The gel was then placed in an Electronic U.V. Transilluminator to detect the presence a 1500 bp band. The size of the DNA fragments was estimated using a FastGene 100 bp DNA Ladder (Nippon Genetics, Germany). A Fast Gene™ Gel/PCR Extraction kit (Nippon Genetics, Germany) was used for purification before sending the extracted DNA for sequencing, according to the manufacturer's instructions. The amplicons were sequenced by sanger-sequenced at Gifu University, Japan, using a multi-capillary DNA sequencer (ABI Prism 3100 or 3130 Genetic Analyzer; Thermo Fisher Scientific Inc., Waltham, MA, USA). An average of 700 bp for each sequence, from each side, was read and compared against the NCBI database using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Four isolates had been chosen from VCO. Isolates with 98 % or higher similarity in sequences were identified as the same species.

2.2.2.3 Screening Antibacterial Activity

Antibacterial activity was estimated by a disk diffusion agar test, based on the presence of clear halos. LAB were grown at 37 ˚C for 24 h in MRS broth, centrifuged for 5 min at 8000×g and supernatant filtered at a pore size of 0.20 µm (Advantec MFS, Inc., Japan). *Escherichia coli* K12 JM109*, Bacillus subtilis* and *Staphylococcus aureus* JCM 20624 were used as indicator bacteria and grown in Luria Bertani (LB) broth (Becton, Dickinson and Company, USA) at 30˚C for 24 h. Cell-Free Supernatant (CFS) from LAB (100 µL) was transferred to wells on Muller Hilton Agar (Becton, Dickinson and Company, USA). Plates were incubated for 24 h at 30 °C to investigate the antibacterial activity of the supernatants by halo formation (zone of growth inhibition). Sodium ampicillin (Wako Pure Chemical Industries, Japan) 100 µg/mL was used as a positive control. Isolates with clear zones of growth inhibition with a diameter > 1 mm around wells were considered as positive. The best isolate from this antibacterial screening was continued to the next stage of the experiment.

2.2.2.4 Effect of Saline Stress Conditions, pH, and Temperature on Antibacterial Activity

L. plantarum COY 2906 was grown at 37 ˚C for 24 h in MRS broth. CFS samples were incubated at temperatures of 60, 70, 80, 90, and 100 °C for 30 min. *L. plantarum* COY 2906 was cultured in MRS broth under different pH conditions: 3, 4.5, 7.5, and 9 at 37 ˚C, for 24 h. The pH was adjusted using 5 mol/L NaOH (Wako Pure Chemical Industries, Japan) for increasing the pH and 5 mol/L of HCl to decrease the pH. To investigate the effect of salinity, *L. plantarum* COY 2906 was cultured in MRS Broth with 4, 6, and 8 % NaCl (Wako Pure Chemical Corporation, Japan) at 37 ˚C for 24 h. The antibacterial activity of CFS samples was estimated by a disk diffusion agar test based on the presence of a clear halo. *E. coli* K12 JM109*, B. subtilis* and *S. aureus* JCM 20624 were used as indicator bacteria.

2.2.2.5 Effect of Saline Stress Conditions, pH, and Temperature on Lactic Acid and Plantaricin Production

2.2.2.5.1 Lactic Acid Determination

Lactic acid was determined using a protocol based on the spectrophotometric determination of lactic acid [41] using the CFS of *L. plantarum* COY 2906 and $FeCl_3.6H_2O$ (Wako Pure Chemical Industries, Japan). Lactic acid (Wako Pure Chemical Industries, Japan) was used as a standard, with the equation of the calibration curve: $y = 0.5969x + 0.6972$. The correlation coefficient of 0.979 was determined using spectrophotometry (Molecular Device SpectraMax M5, US).

2.2.2.5.2 Identification of Plantaricin Genes from *L. plantarum* COY 2906 Using Specific Primers

Identification of plantaricin was performed using PCR with total DNA of *L. plantarum* COY 2906 as a template. Specific primers of the bacteriocin used in this study are shown in Table 1.

Table 1. PCR primer sequences

Name	Sequence (5′ → 3′)	Annealing	References
plnA_F	TAGAAATAATTCCTCCGTACTTC	55	[1]
plnA_R	ATTAGCGATGTAGTGTCATCCA		
plnEF_F	TATGAATTGAAAGGGTCCGT	54	
plnEF_R	GTTCCAAATAACATCATACAAGG		
plnS_F	GCCTTACCAGCGTAATGCCC	56	[25]
plnS_R	CTGGTGATGCAATCGTTAGTTT		
plnC8α_F	CGGGGTACCGATGATGATGATAAAG	56	[42]
plnC8α_R	CATGCCATGGCTAAAATTGAACATA		
plnC8β_F	CGGGGTACCGATGATGATGATAAAT	56	
plnC8β_R	CATGCCATGGTTAATGATAAAAGCCTT		
plnNJK_F	CTAATAGCTGTTATTTTTAACC	55	[26]
plnNJK_R	TTATAATCCCTTGAACCACC		
pln423A_F	GTCGCCCGGGAAATACTATGGTAATGGGG	58	[27]
pln423A_R	GCGTCCCGGGTTAATTAGCACTTTCCATG		
plnZJ5_F	AGATTCCAGGCAATG	55	[43]
plnZJ5_R	GGAATAAATCAGTTA		

2.2.2.6 Copy Number of Plantaricin Plasmid Genes of *L. plantarum*

The copy numbers of plantaricin genes, *plnA, plnEF, plnN, plnJ,* and *plnk* of *L. plantarum* COY 2906 were determined by Real Time qPCR (ABI Step One Plus, Thermo Fisher Scientific Inc.) on total DNA. Real Time qPCR was performed with 10 µL final volume containing 1 µL of total DNA template, 1 µL of each primer at a concentration of 0.5 µM (Table 2), 2 µL of RNAse free-water, and 5 µL of Power SYBR® Green PCR Master Mix (Applied Biosystems, Thermo Fisher Scientific, UK) with an initial step at 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s, 60 °C for 1 min and 72 °C for 30 s. *16S rRNA* was selected as housekeeping genes on the basis of the available literature; *plnA, plnEF, plnN, plnJ*, and *plnK* were selected on the basis of a screening test for plantaricin-encoding genes of the strain used. Results were analyzed using the comparative critical threshold method (ΔCT) in which the amount of target genes was adjusted to a reference (housekeeping gene) [44]. They were taken in triplicate for each gene.

Table 2 Real Time PCR primer sequence and amplicon size to determine relative amounts of plantaricin plasmid genes

Target Genes	Sequence	Amplicon size	Reference
Housekeeping			
16S rRNA_F	GATGCATAGCCGACCTGAGA	114	[38]
16S rRNA_R	CTCCGTCAGACTTTCGTCCA		
Plantaricins			
plnA_F	AAAATTCAAATTAAAGGTATGAAGCAA	108	
plnA_R	CCCCATCTGCAAAGAATACG		
plnEF_F	GTTTTAATCGGGGCGGTTAT	85	
plnEF_R	ATACCACGAATGCCTGCAAC		[44]
plnN_F	GCCGGGTTAGGTATCGAAAT	102	
plnN_R	TCCCAGCAATGTAAGGCTCT		
plnJ_F	TAAGTTGAACGGGGTTGTTG	102	
plnJ_R	TAACGACGGATTGCTCTGC		
plnK_F	TTCTGGTAACCGTCGGAGTC	97	
plnK_R	ATCCCTTGAACCACCAAGC		

2.2.2.7 Sequencing Draft Genome *L. plantarum* COY 2906 isolated from VCO

A genomic DNA sample from *L. plantarum* COY 2906 was sequenced on an Illumina MiSeq platform (Illumina Corporation, San Diego, CA, USA) with 300 bp paired-end reads by the Gifu University Next Generation Sequencer service. Raw sequence data was obtained as FASTQ files, and FASTQ Quality Trimmer Galaxy Version 1.1.1 was used to trim the reads using default parameters. Quality-filtered reads were assembled into contigs using the SPAdes function of the online tool Galaxy (https://usegalaxy.org/) with default parameters. SPAdes and BayesHammer/IonHammer were used, along with our own read correction tool, which can be used to turn the error correction module off during the read error correction stage, to obtain high-quality assemblies. The draft genome was annotated using DFAST (https://dfast.nig.ac.jp/).

2.2.3 Results

2.2.3.1 *Lactobacillus* spp. were isolated from VCO

The Isolation of LAB was done to investigate *Lactobacillus* spp. activities under several stressor treatments. The production of lactic acid and the multiplicity of plantaricin plasmid genes were evaluated to find out the correlation between each stressor and the production of antimicrobial substances. Four LAB candidates were isolated from VCO. The total bacterial load of VCO was 3.4 x 10^8 CFU/mL. The 16S rRNA for the LAB isolates was amplified using universal primers, to confirm the species, and the result showed a length of 1500 bp. All four candidates were sequenced, and we confirmed that three colonies showed 99 % similarity with *L. plantarum* and one colony showed 99 % similarity with *L. sakei*.

2.2.3.2 *L. plantarum* COY 2906 shows greater inhibition than *L. sakei*

We screened for the best inhibitor of indicator bacteria, for later use in the experiments which followed. *L. plantarum* COY 2906 and *L. sakei* were screened for their antibacterial activity. They were harvested at the beginning of stationary phase. The zones of inhibition of *L. plantarum* COY 2906 and *L. sakei* against the indicator bacteria are shown in Figure 1. Based on this result, *L. plantarum* COY 2906 and *L. sakei* can inhibit *E. coli* K12 JM109*, B. subtilis* and, *S. aureus* JCM 20624. However, both had their greatest effect on *S. aureus* JCM 20624. Sodium ampicillin 100 µg/mL was used as positive control. *L. plantarum* COY 2906 showed stronger inhibition than *L. sakei*. Therefore, *L. plantarum* COY 2906 was selected for further analysis.

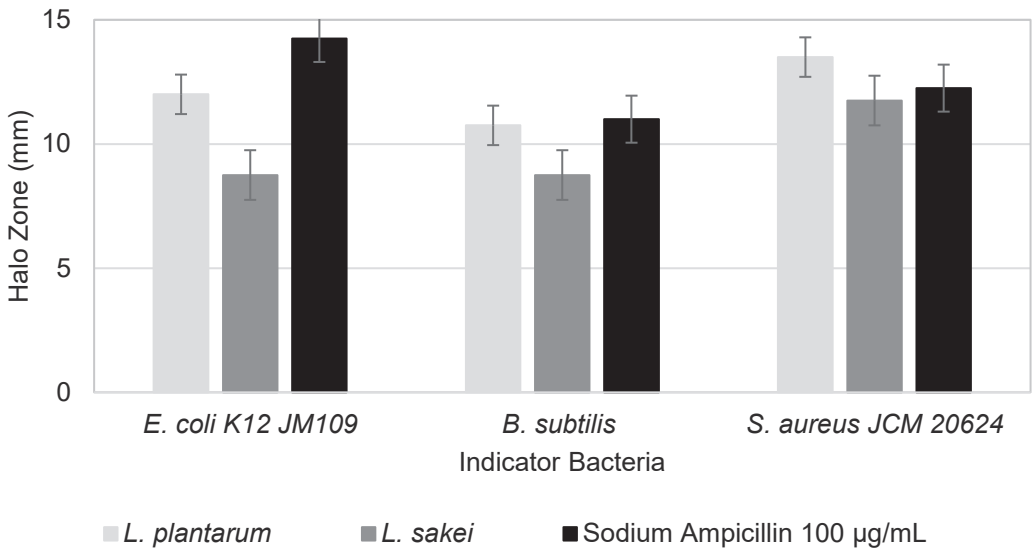

Fig. 1. The inhibition zones of *L. plantarum* and *L. sakei* against the indicator bacteria

2.2.3.3 Antimicrobial Activity of *L. plantarum* COY 2906 with Saline Stress, pH and High Temperature

The antimicrobial activity of *L. plantarum* COY 2906 was measured under saline stress conditions, and for a range of pH and temperature treatments. The results of the measurement of halo zones, were as follows: under saline stress conditions *L. plantarum* COY 2906 did not show activity against *E. coli* K12 JM109, and was better at inhibiting *B. subtilis* than *S. aureus* JCM 20624. With 4 % and 6 % NaCl, *L. plantarum* COY 2906 showed more inhibition of *B. subtilis* that it did without salt. However, the inhibition of *S. aureus* JCM 20624 was the greatest without salt (see Table 3).

Table 3 The inhibition zone of *L. plantarum* under several stressor

Treatment	*E. coli K12 JM10* (mm)	*B. Subtilis (mm)*	*S. aureus JCM 20624 (mm)*
Without Treatment (0% NaCl, pH 6.3)	12 ± 0.35	10.75 ± 0.35	13.5 ± 0.35
Saline Stress			
4% NaCl	0	13.25 ± 0.35	10.25 ± 2.12
6% NaCl	0	11.25 ± 1.06	8.875 ± 2.30
8% NaCl	0	9	5.375 ± 0.88
Acidity Stress			
pH 3	5.75 ± 0.35	5.75 ± 0.35	0
pH 4.5	9.25 ± 0.35	8.75 ± 0.35	7.25 ± 0.35
pH 7.5	9 ± 0.70	8	7 ± 0.70
pH 9	4.5	6.75 ± 0.35	6.5 ± 0.70
Temperature			
T = 60°C	NT	8.5 ± 0.70	NT
T = 70°C	NT	7.5	NT
T = 80°C	NT	7.5	NT
T = 90°C	NT	7.5	NT
T = 100°C	NT	7.5	NT

NT: Not Tested

Standard deviation with n=2

Under alkaline and acid stress, the inhibition zone was smaller for all indicator bacteria compared to the control (pH 6.3), and at pH 3.0, *L. plantarum* COY 2906 showed no inhibition of *S. aureus* JCM 20624. The optimum inhibition was between pH 4.5 and 7.5 for all indicator bacteria, as seen in Table 3, with the peak of inhibition at pH 4.5. The only indicator species used in our elevated temperature study was *B. subtilis.* This was done because of the sensitivity of the two other species, and the temperature had little effect on inhibitory activity, which stayed fairly high, at temperatures from 60 to 100 ^{o}C.

2.2.3.4 *L. plantarum* COY 2906 produced Lactic Acid and Plantaricin as Antimicrobial Compounds

2.2.3.4.1 Lactic Acid Production Under Acidity and Saline stress

Lactic acid is an organic compound that can be bactericidal and bacteriostatic to indicator bacteria. It was measured to find the effect of saline stress conditions and pH on its production. In the growth phase, the lactic acid concentration increased from 0.4 % to 3.2 % after 24 h incubation, and then decreased to 2.9 % at 72 h, without treatment. However, under saline conditions of 4 %, 6 % and 8 % NaCl, lactic acid concentration increased from 0.8 %, 0.3 % and 0.4 % to become 2.9 %, 2.8 % and 2.1 %, respectively, after 72 h incubation (Figure 2). From these data, lactic acid production and saline contain were inversely proportional; a culture with high saline concentration will reduce lactic acid production.

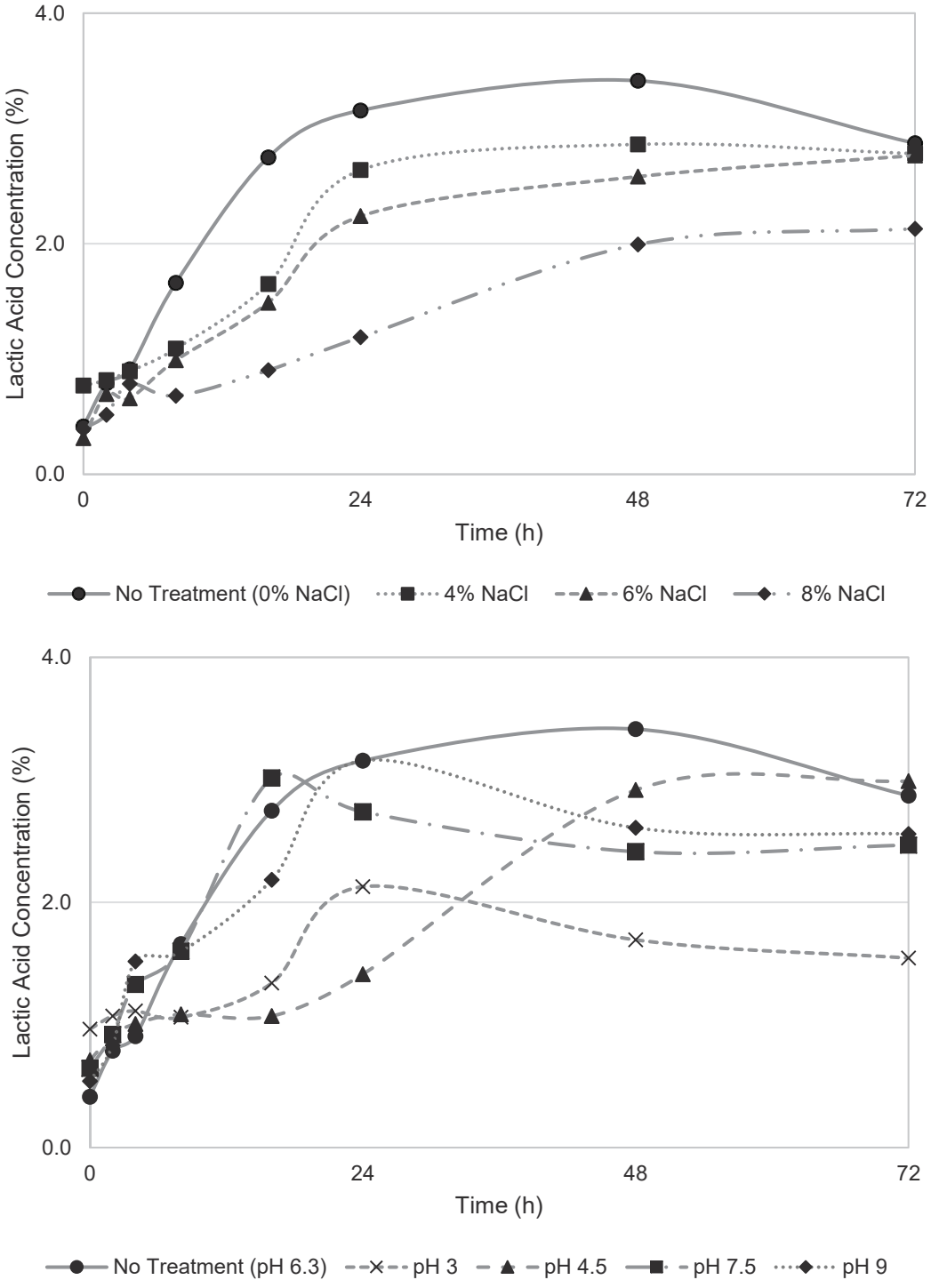

Fig. 2 Correlation between lactic acid production under saline and acidity stress. (A) Lactic acid production under saline stress. (B) Lactic acid production under pH stress

Figure 2 shows that lactic acid production will decrease in conditions of pH stress. This is seen by the differences in value between the beginning and the end of each trial. At pH 3, *L. plantarum* COY 2906 produced less lactic acid than others. However, alkaline conditions (pH 7.5 and 9) will make *L. plantarum* COY 2906 produce more lactic acid than in acid conditions (pH 3 and 4.5).

2.2.3.4.2 *L. plantarum* COY 2906 Encoded Plantaricin

The genes encoding bacteriocin production were identified by detecting the presence of plantaricin genes in the total DNA of *L. plantarum* COY 2906*,* using PCR. The result of gel separation showed the presence of *plnA*, *plnEF*, and *plnNJK, at* ~550 bp, ~550 bp, and ~1500 bp, respectively (see Fig. 3). Genes *plnS, plnC8α, plnC8β, pln423A,* and *plnZJS* were not present, based on observation of the PCR product.

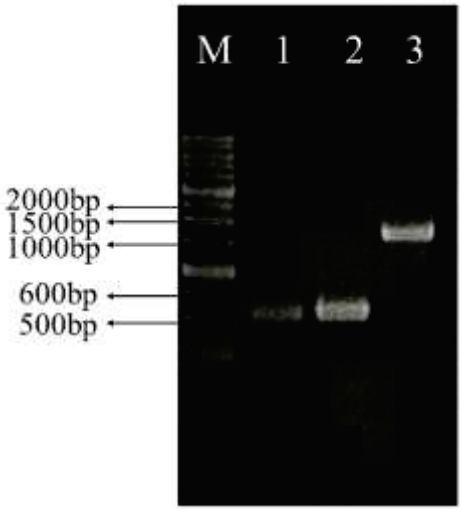

Fig. 3 Detection of plantaricin genes in total DNA of *L. plantarum.* M: molecular weight marker (100 bp DNA ladder). 1. *plnA*, 2. *PlnEF*, 3. *plnNJK*

2.2.3.5 Multiplication of Plantaricin Genes on Plasmid of *L. plantarum* COY 2906 under pH and Saline Stress Conditions

The multiplication of plantaricin genes was measured in order to see the effect of the stressors on their production. The genes studied were *plnA, plnEF, plnN, plnJ* and *plnK*. The relative number was calculated in the beginning of the stationary phase in order to determine the genes responsible for bioactivity. Figure 4 presents the relative numbers of *plnA, plnEF, plnN, plnJ* and *plnK*.

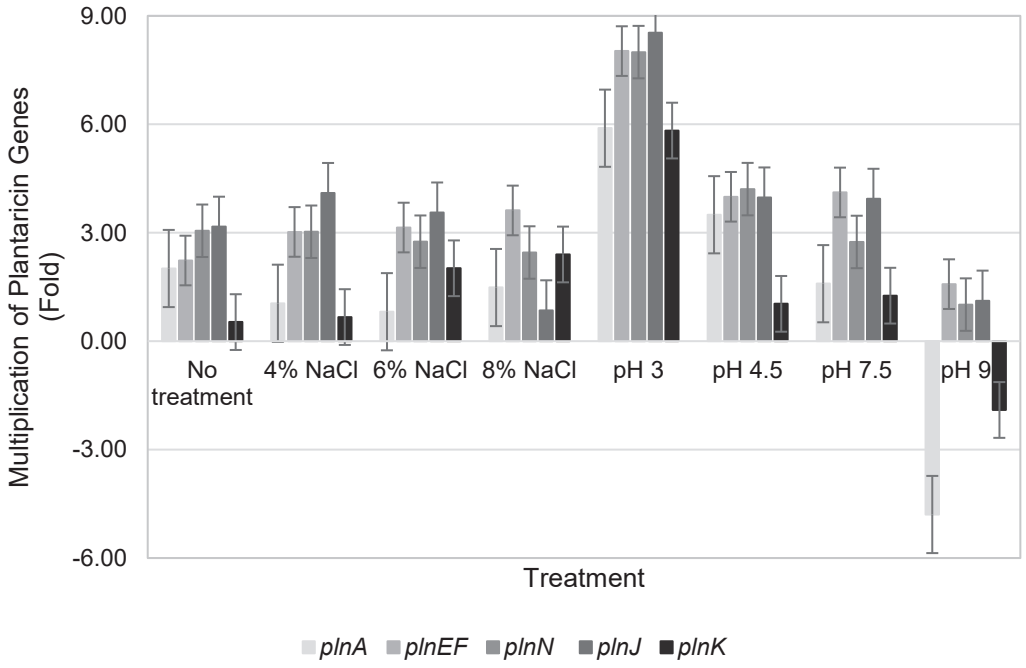

Fig. 4 Multiplication Plantaricin Genes on Plasmid of *L. plantarum* under Acidity and Saline Stress Conditions

From Figure 4, it can be concluded that multiplication of *plnA* gene on plasmid is not affected by salinity. Multiplication of *plnEF* and *plnK* genes on the plasmid appear to increase and *plnN* and *plnJ* decrease slightly in saline conditions. However, the copy number of *plnA* and *plnK* significantly increased in highly acidic conditions (pH 3) and in alkaline conditions (pH 9) underwent a loss in number from the initial level. Multiplication of *plnEF*,

plnN, and *plnJ* genes on the plasmid will also increase at pH 3 and be lower in alkaline conditions (pH 9) compared to the control.

2.2.3.6 Plantaricin Genetic Analysis of *L. plantarum* COY 2906 Genome

The draft genome was sequenced by MiSeq for Illumina next-generation sequencing, which can analyze hundreds of genes simultaneously from 12 to more than 24,000 amplicons in a single panel. Draft genome sequencing and comparative genomic analysis could provide functional information. The functional information will provide all the plantaricin loci on the chromosome and provide information about supporting material such as immunity protein, secretion genes, or histidine protein kinase, and this information will highlight the amino acid differences between our sample and GenBank data. The draft genome of *L. plantarum* COY 2906 is composed of one 3.26 Mbp circular genome (see Supporting Table 4 for detail).

Table 4. Genome Features of *L. plantarum*

Attribute	**Value**
Genome Size (bp)	3,269,379
GC Content (%)	44.80%
CDSs	3,008
rRNA	1
tRNA	42
CRISPRS	0

Plantaricins are bioactive peptides or proteins produced by *L. plantarum* COY 2906*,* displaying antimicrobial activity against other bacteria. The genome of *L. plantarum* COY 2906 consists of several encoding genes involve in plantaricin production (Figure 5): *plnW (Locus_28580), plnV (Locus_28570), plnU (Locus_28560), plnH (Locus_28540), plnG (Locus_28530), plnE (Locus_28520), plnF (Locus_28510), plnD (Locus_28490), plnC*

(Locus_28480), plnB Locus_28470), plnA (Locus_28460), plnR (Locus_26530), plnL (Locus_26520), plnK (Locus_26510), plnJ (Locus_26500), plnM (Locus_26490), plnN (Locus_26480), plnO (Locus_26470), and *plnP (Locus_26460).* This draft genome sequence lends insight into the genetic elements involved in antimicrobial activity. Moreover, it is suggested that the activity of *L. plantarum* COY 2906 against pathogens is associated with the plantaricin biosynthesis gene cluster.

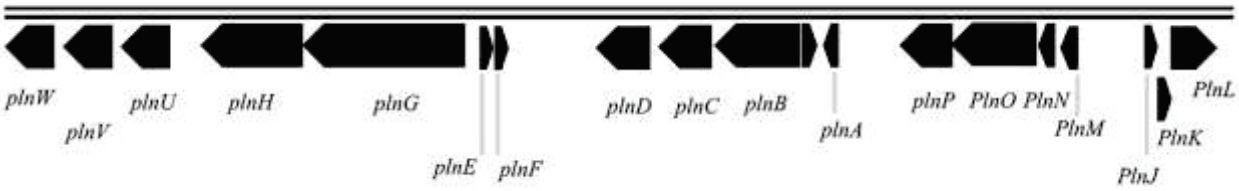

Fig. 5 Genetic graph of plantaricin biosynthesis gene cluster of *L. plantarum*

2.2.4 Discussion

Isolation of LAB was done to investigate the biological role of *Lactobacillus* in VCO fermentation products. The production of lactic acid and bacteriocin from isolates is characterized as bio-preservative; to prevent food damage from pathogenic bacteria and extend shelf life. *L. plantarum* COY 2906 and *L. sakei* were isolated from VCO. *L. plantarum* COY 2906 showed stronger inhibition than *L. sakei* of indicator bacteria *E. coli* K12 JM109*, B. subtilis* and *S. aureus* JCM 20624. *L. plantarum* COY 2906 was chosen for further analysis of lactic acid production and bacteriocin genes.

L. plantarum COY 2906 inhibited *B. subtilis* and *S. aureus* JCM 20624 under saline stress conditions but showed less inhibition and less lactic acid production. At pH 3, *L. plantarum* COY 2906 did not inhibit *S. aureus* JCM 20624 and the optimum pH range for inhibiting indicator bacteria was 4.5 – 7.5. Wu (2013) reported that cell growth, glucose utilization, and lactic acid production of *Lactococcus lactis* NZ9000 and *Lactobacillus casei* RecO decreased when cultured with 3 % NaCl. However, this decrease was found to be relatively minor in comparison to the efficiency of nisin-inducible RecO expression in increasing lactate productivity [45]. We can conclude that, in saline conditions, antimicrobial activity and lactic acid production decreases and, under pH stress conditions, the optimum condition to produce lactic acid is at pH 4.5 – 6.3. However, alkaline conditions will make *L. plantarum* COY 2906 produce more lactic acid than in acid conditions. Alkalinity stimulates lactic acid production and high saline content reduces it. It has been found that environmental changes also affect amylase production in *L. plantarum*. Amylase production was optimal at pH 6.0, and it has been reported that pH_{in} can regulate various metabolic functions; it is possible that a decrease in intracellular pH interferes with amylase synthesis or secretion [46].

Lactic acid produced by *Lactobacillus* was reported to disrupt the membrane of *Campylobacter jejuni*, as measured by biophotonics [47]. Lactic acid can generate hydroxyl

radicals, depending on intracellular iron ions, change cell membrane permeability, cause DNA damage and cell death [48]. The presence of lactic acid is expected to kill or inhibit pathogenic bacteria; the pH stress results showed that there is correlation between the inhibitory activity and lactic acid production.

L. plantarum COY 2906 produces bacteriocin, encoded on plasmid and chromosome. The presence of *plnA*, *plnEF* and *plnNJK,* in an isolate of *L. plantarum* COY 2906 obtained from our VCO sample, were confirmed by observation of the PCR product, using specific primers. Under saline stress conditions, the multiplication of plantaricin plasmid genes (*plnA, plnEF, plnN, plnJ,* and *plnK)* were not changed significantly, but there was significant change under acid stress. At pH 3, the relative number of plantaricin genes increases sharply. However, it decreases in alkaline conditions. Under acidic conditions, plantaricin encoded on plasmids replicates freely and helps with bacteriostatic and bactericidal function.

However, bacteriocin production by *L. amylovorus* DCE 471 was reported to be higher at pH 5.4 than 6.4, at 37 °C, in the secondary growth phase, and decreased with 3 % NaCl at pH 5.4 compared with a control [49]. Plantaricin genes encoded on the plasmid were supposedly replicated more under acid stress than in normal pH (pH 6.3). This genetic control serves mainly to allow the cell to adjust to changes in its nutritional environment, so that its main function of growth and division can be optimized. In bacteria, as ribosomes begin translating nascent mRNA as soon as the first ribosome-binding site is assembled, it follows that initiation of transcription is a critical point of gene control in bacterial cells (Darnell, Lodish, & Baltimore, 1986). In our study, increasing the number of plantaricin genes on the plasmid, in acidic conditions, is indicative of antimicrobial activity.

The draft genome sequence and comparative genomic analysis of our strain could provide some functional analysis to gain an insight into genetic elements involved in antimicrobial activity. Antimicrobial activity of *L. plantarum* COY 2906 against pathogens has

been associated with the plantaricin biosynthesis gene cluster and its product. Plantaricin A precursor peptide, potentially encoded by *plnA,* could induce the transcription of the following genes, organized into operons: *plnW, plnV, plnU, plnH, plnG, plnE, plnF, plnD, plnC, plnB, plnR, plnL, plnK, plnJ, plnM, plnN, plnO,* and *plnP.* Genes *plnEF* and *plnJK* code for a pair of peptides bacteriocins, which are 10^3 times more active when combined with their complementary peptide than individually [21][50]. Plantaricin plays the major anti-bacterial role against pathogenic bacteria in VCO fermentation products, extending their shelf life and preventing food damage.

The draft genome sequence allows a better understanding, at a molecular level, of antimicrobial activity and probiotic potential, and could facilitate the protection of dairy products to become safer, with potential health benefits. The activity of Class IIb bacteriocins, such as *plnEF* and *plnJK*, depends on the overall activity of both peptides [51], acting synergistically. The activity of both peptides is greater than the effect of each peptide, separately [1].

2.2.5 Conclusion

In conclusion, plantaricin and lactic acid have the key role in preventing from spoilage and extending its shelf life. Although these antimicrobial activities are relatively well understood, its role in VCO fermentation products would be interesting to study. This research clearly showed that lactic acid production and the multiplication of plantaricin genes are affected by salinity and pH. These each play an important role in extending the shelf life of the product and preventing bacterial damage to food.

2.3 Role of Plantaricin Produced by *Lactobacillus plantarum* on Fermentation of Ishizuchi-kurocha

2.3.1 Introduction

Post-fermented tea is one of the most important beverages in East Asian countries, such as Thailand, Myanmar, China, and Japan. This type of tea is produced via fermentation by microbes. In Japan, four traditional post-fermented teas are commonly consumed: Ishizuchi-kurocha (Ehime), Goishi-cha (Kochi), Awa-bancha (Tokushima), and Batabatacha (Toyama). Ishizuchi-kurocha is produced by a two-step fermentation process, i.e., primary and secondary fermentation [52]. During primary fermentation, the tea leaves are fermented by fungi, such as *Aspergillus niger*, under aerobic conditions for approximately one week, and during secondary fermentation, the tea leaves are fermented by *Lactobacillus* spp., such as *L. plantarum*, under anaerobic conditions for approximately two weeks [53][54]. *Klebsiella pneumoniae* subsp. *ozaenae*, *Pseudomonas glareae*, *K. variicola*, and *P. aeruginosa* have been observed on tea leaves before fermentation [63], and these bacteria can be opportunistic pathogens of humans, causing lung infections [55], cystic fibrosis, and infections of traumatic burns [56]. *L. plantarum* is one of the major lactic acid bacteria species present on tea after primary and secondary fermentation [52], and it exerts *in vitro* anti-microbial effects on several potentially pathogenic species, such as *Listeria monocytogenes*, *Bacillus cereus*, *Escherichia coli*, *Yersinia enterocolitica*, *Citrobacter freundii*, *Enterobacter cloacae*, *Enterococcus faecalis*, *Salmonella enterica* subsp. *enterica*, and *Candida albicans* [3]. *L. plantarum* produces antimicrobial substances, such as organic acids, including lactic and fatty acids, and bacteriocins. Bacteriocins are proteinaceous antibiotics that exert bactericidal and bacteriostatic effects on bacteria that are closely related to the bacterial species by which they are produced [15]. The bacteriocins produced by *L. plantarum* are known as plantaricins, which are categorized as class II bacteriocins. This class of plantaricins is very large, and the mechanism by which bacteria

are killed or inhibited differ among plantaricins. Class II bacteriocins are small (<10 kDa), heat-stable molecules with amphiphilic helical structures that enable insertion into the cytoplasmic membrane of the target cell, thereby causing membrane depolarization and cell death [2]. They are non-antibiotics or non-modified or pediocin-like antibiotics with isoelectric points in a range from 8.3 to 10.0 [3].

The genes encoding bacteriocins are located in operon clusters, and may be present on the chromosome, e.g., PlantaricinST31 [3], or on a plasmid, e.g., Plantaricin423 [57]. These genes may also occur in transposons, e.g., Nisin [58]. We examined the role of the bacteriocin genes of *L. plantarum* during the processing of Ishizuchi-kurocha tea. This study focused on the plantaricin genes in the bacterial genome and on plasmids and their effects on fermented tea processing.

2.3.2 Materials and Methods

2.3.2.1 Isolation and Identification of Lactic Acid Bacteria

L. plantarum strain IYO1511 was previously isolated from Ishizuchi-kurocha. Strain IYO1501 was newly isolated from Ishizuchi-kurocha and was identified as *L. plantarum* by the degree of similarity between its 16S rRNA gene and that of strain IYO1511 [52]. *L. plantarum* strains were grown in Man, Rogosa, and Sharpe (MRS) broth at 37°C for 18 h.

2.3.2.2 Preparation of Plasmids from *L. plantarum* Strains from Fermented Tea

Plasmids were isolated according to a protocol based on *Lactobacillus* spp.-oriented high-quality methods [23], with some modifications. Briefly, *L. plantarum* was cultured in 2 mL of MRS broth for 18 h. Then, the culture was centrifuged at 14,000 × *g* for 5 min. The supernatant was removed, and the cell pellet was resuspended in 200 µL of a 25% sucrose solution containing 30 mg/mL lysozyme. Next, 200 µL of 0.5 M EDTA was added to the solution and incubated at 37°C for 15 min. After incubation, 400 µL of alkaline sodium dodecyl sulfate (SDS) solution (3% SDS in 0.2N NaOH) was added, and the mixture was incubated at 27°C for 7 min. Next, 300 µL of ice-cold 3 M sodium acetate (pH 4.8) was added, mixed immediately, and centrifuged at 14,000 × *g* and 4°C for 15 min. The supernatant was transferred to a new tube, and 650 µL of isopropanol (R/T) was added. The solution was immediately mixed and centrifuged at 14,000 × *g* for 15 min at 4°C. All the liquid was removed, and the pellet was resuspended in 320 µL of sdH_2O, and 200 µL of 0.5M EDTA, 200 µL of 7.5 M ammonium acetate, and 350 µL of phenol:chloroform (1:1) were added. The solution was mixed immediately and centrifuged at 14,000 × *g* and 27°C for 5 min. The top aqueous phase was transferred to a new tube, and 1 mL of ethanol was added. The solution was mixed immediately and centrifuged at 14,000 × *g* and 4°C for 15 min. The supernatant was discarded, and the pellet was resuspended in 40 µL of TE buffer.

2.3.2.3 DNA Isolation from *L. plantarum* and Fermented Tea

Total bacterial DNA was extracted from the *L. plantarum* strains and tea leaf samples using the Extrap Soil DNA Kit Plus, ver. 2 (Nippon Steel & Sumikin Eco-Tech Corporation, Japan) according to the manufacturer's instructions. The quality of the extracted DNA was assessed by gel electrophoresis using a 1% agarose gel in 1× TAE buffer, and the gel was photographed under UV light.

2.3.2.4 Identification of Genes Encoding Bacteriocins in *L. plantarum* and Fermented Tea

Genes encoding bacteriocins were identified by PCR. The PCR primers used are shown in Table 1. Plasmid and total DNA from *L. plantarum* and total DNA from nine samples of fermented tea were used as templates for amplification. Plantaricin amplicons were Sanger-sequenced at Gifu University, Japan, using a multi-capillary DNA sequencer (ABI Prism 3100 or 3130 Genetic Analyzer; Thermo Fisher Scientific Inc., Waltham, MA, USA). *L. plantarum* COY 2906 isolated from virgin coconut oil, which was previously been shown to contain plantaricin genes, was used as a positive control.

Table 1. PCR primer sequences

Name	Sequence (5′ → 3′)	Annealing	References
plnA-F	TAGAAATAATTCCTCCGTACTTC	55	[1]
plnA-R	ATTAGCGATGTAGTGTCATCCA		
plnEF-F	TATGAATTGAAAGGGTCCGT	54	
plnEF-R	GTTCCAAATAACATCATACAAGG		
plnS_F	GCCTTACCAGCGTAATGCCC	56	[25]
plnS_R	CTGGTGATGCAATCGTTAGTTT		
plnC8α-F	CGGGGTACCGATGATGATGATAAAG	56	[42]
plnC8α-R	CATGCCATGGCTAAAATTGAACATA		
plnC8β-F	CGGGGTACCGATGATGATGATAAAT	56	
plnC8β-R	CATGCCATGGTTAATGATAAAAGCCTT		
plnNJK_F	CTAATAGCTGTTATTTTTAACC	55	[26]
plnNJK_R	TTATAATCCCTTGAACCACC		
pln423A_F	GTCGCCCGGGAAATACTATGGTAATGGGG	58	[27]
pln423A_R	GCGTCCCGGGTTAATTAGCACTTTCCATG		
plnZJ5_F	AGATTCCAGGCAATG	55	[43]
plnZJ5_R	GGAATAAATCAGTTA		

2.3.2.5 Draft Genome of *L. plantarum* IYO1511 Isolated from Post-fermented Tea

A genomic DNA sample from *L. plantarum* IYO1511 was sequenced on an Illumina MiSeq platform (Illumina Corporation, San Diego, CA, USA) with 300 bp paired-end reads by the Gifu University Next Generation Sequencer service. Raw sequence data was obtained as FASTQ files, and FASTQ Quality Trimmer Galaxy Version 1.1.1 was used to trim the reads using default parameters. Quality-filtered reads were assembled into contigs using the SPAdes function of the online tool Galaxy (https://usegalaxy.org/) with default parameters. SPAdes and BayesHammer/IonHammer were used, along with our own read correction tool, which can be used to turn the error correction module off during read error correction stage, to obtain high-quality assemblies. The draft genome was annotated using DFAST (https://dfast.nig.ac.jp/).

2.3.2.6 Antibacterial Effects of *L. plantarum* Strains

The antibacterial effects of the strains were estimated using the disk diffusion method [22], and the results were evaluated based on the presence of a clear halo surrounding the disk. *L. plantarum* strains were grown in MRS broth at 37°C for 12 h and then centrifuged at 8,000 × *g* for 5 min. The supernatant was filtered through a 0.20-μm pore size filter (Advantec MFS, Inc., Japan) and used for the assay. *Escherichia coli* K12 JM109, *Bacillus subtilis*, and *Staphylococcus aureus* JCM 20624, which were used as the indicator bacteria, were grown in Luria Bertani broth (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) at 30°C for 12 h. *L. plantarum* COY 2906, which is known to express plantaricin, was used as a positive control. The disks were placed on Mueller-Hinton agar (Becton, Dickinson and Company) and were impregnated with 100 μL of cell-free supernatant of the *L. plantarum* strains. Plates were incubated at 30°C for 24 h to assess antibacterial activity as halo formation (i.e., the zone of growth inhibition). Isolates that produced clear zones, indicating growth inhibition, with a diameter >1 mm were considered positive.

2.3.2.7 Fermented Tea Sample Collection

Nine samples of Ishizuchi-kurocha tea were collected from three manufacturers in 2017 (Table 2) and stored at -80°C. Details of the samples were reported previously [2].

Table 2. Ishizuchi-kurocha tea samples collected from three manufacturers in Ehime, Japan

Producer	Process of fermentation	Code Number
A	Before Fermentation	1
	Primary Fermentation	2
	Secondary Fermentation	3
B	Before Fermentation	4
	Primary Fermentation	5
	Secondary Fermentation	6
C	Before Fermentation	7
	Primary Fermentation	8
	Secondary Fermentation	9

2.3.3 Result

2.3.3.1 *L. plantarum* Isolated from Post-fermented Tea Expresses Plantaricin

L. plantarum was the predominant lactic acid bacteria species present during primary and secondary fermentation of Ishizuchi-kurocha tea. Therefore, the plantaricin genes of *L. plantarum* IYO1501 and IYO1511 were analyzed. PCR-based identification of the genes encoding this bacteriocin was performed to detect these genes on the plasmids and chromosomes of *L. plantarum* IYO1501 and *L. plantarum* IYO1511. Cells were harvested at early stationary phase, when plantaricin production is maximal.

The presence of the *plnA* and *plnEF* genes on plasmids was confirmed by PCR amplification of 550 bp products using primers specific for *plnA* and *plnEF* (Fig. 1).

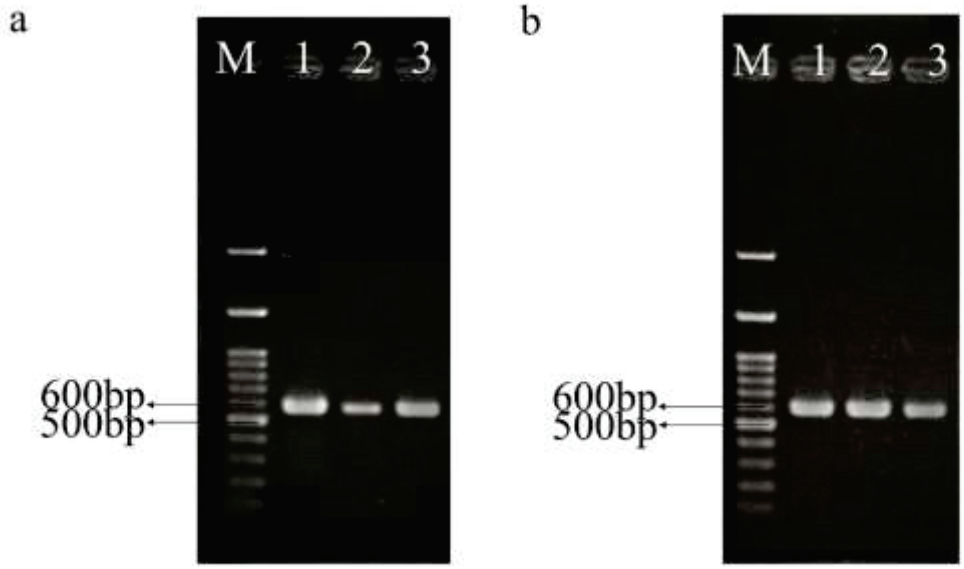

Fig. 1 Detection of plantaricin genes on *L. plantarum* plasmids. **a.** Detection of *plnA*, **b.** detection of *plnEF*. M: molecular weight marker (100 bp DNA ladder). 1: Positive control, amplification of plantaricin gene fragments from *L. plantarum* COY 2906, 2: amplification of plantaricin gene fragments from *L. plantarum* IYO1501, 3: amplification of plantaricin gene fragments from *L. plantarum* IYO1511

The presence of *plnA*, *plnEF*, and *plnNJK* in total DNA was confirmed by PCR amplification of 550, 550, and 1,500 bp products, respectively (Fig. 2). In the total DNA from *L. plantarum* IYO1501, only *plnEF* was detected, and *plnA*, *plnEF*, and *plnNJK* were detected in total DNA from *L. plantarum* IYO1511. *PlnNJK* was not detected in the prepared plasmid DNA from *L. plantarum* IYO1511, suggesting that *plnNJK* is present on the chromosome (Fig. 2). *plnS*, *plnC8α*, *plnC8β*, *pln423A*, and *plnZJS* were not detected by PCR in either the plasmid or total DNA prepared from *L. plantarum* IYO1501 and *L. plantarum* IYO1511.

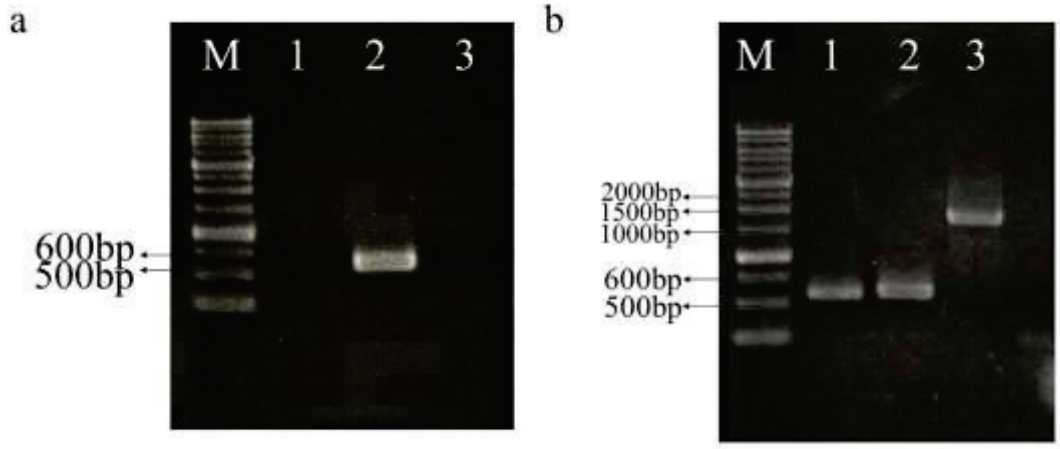

Fig. 2 Detection of plantaricin genes in total DNA from *L. plantarum*. **a.** Detection of plantaricin gene sequences in total DNA from *L. plantarum* IYO1501, **b.** detection of plantaricin gene sequences in total DNA from *L. plantarum* IYO1511. M: molecular weight marker (100 bp DNA ladder). 1. *plnA*, 2. *PlnEF*, 3. *plnNJK*

2.3.3.2 Analysis of the Genome of *L. plantarum* IYO1511

The *L. plantarum* IYO1511 draft genome was deposited in the DDBJ Sequence Read Archive under accession number DRA009644. *L. plantarum* IYO1511 contains bacteriocin genes. The draft genome was sequenced on an Illumina MiSeq platform, which can analyze hundreds of genes simultaneously, with 12 to more than 24,000 amplicons in a single run.

Table 3. Genome characteristics of *L. plantarum* IYO1511

Attribute	**Value**
Genome Size (bp)	3,239,087
GC Content (%)	44.5%
CDSs	3,049
rRNA	2
tRNA	67
CRISPRS	0

Draft genome sequencing and comparative genomic analyses can provide functional information. The genome of *L. plantarum* IYO1511 is circular and 3,23 Mbp long, and detailed characteristics are shown in supplementary Table 3. Analyses were conducted to identify the location of the plantaricin genes on the chromosomes (Fig. 3a) and provide other information regarding immunity proteins, secretion genes, and histidine protein kinases.

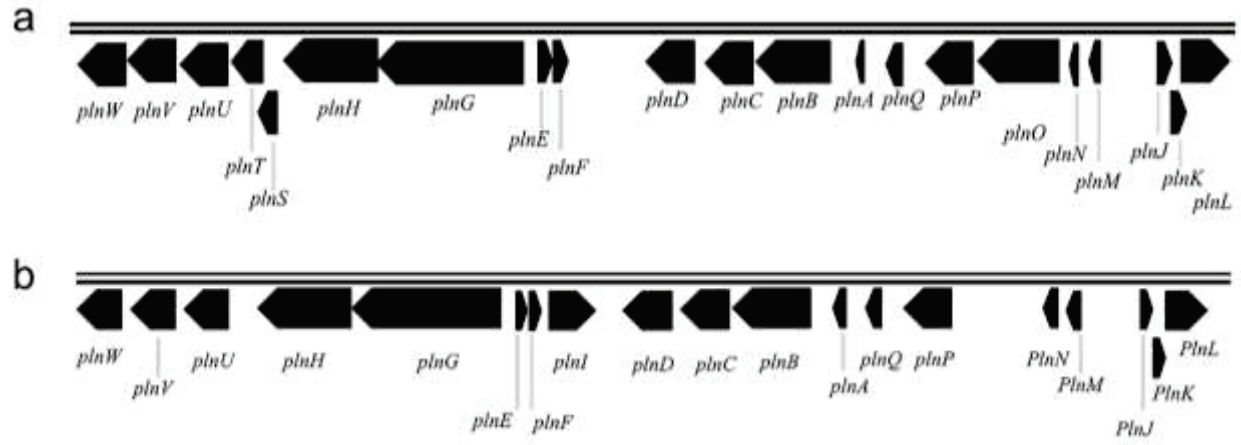

Fig. 3 Plantaricin biosynthesis gene cluster in **a.** *L. plantarum* IYO1511 and **b.** *L. plantarum* WCFS1

The presence of two-component system provides evidence that many biological functions may be mediated through two-component regulation, including bacteriocin biosynthesis. Seven histidine protein kinase and two-component sensor histidine kinase pairs were identified in the genome of *L. plantarum* IYO1511 (Table 4). The genome of *L. plantarum* IYO1511 also contains five genes encoding independent histidine protein kinases (*Locus_00310*, *Locus_07960*, *Locus_08190*, *Locus_17300*, and *Locus_18230*) and a signal transduction histidine kinase encoded by *Locus_28910* (Table 4).

Table 4. Identity of the putative histidine protein kinase observed in *L. plantarum* IYO1511

Gene	Location	Putative Protein Encode
pltK	36432 - 37694	histidine kinase
hpk2_1	Complement (94356 - 95723)	two-component sensor histidine kinase
hpk5	175717 - 177360	two-component sensor histidine kinase
hpk6	100057 - 101160	two-component sensor histidine kinase
hpk11	64510 - 65670	two-component sensor histidine kinase
lamK	164265 - 165515	histidine kinase
hpk9	187091 - 188371	histidine kinase
hpk2_2	Complement (36501 - 37877)	two-component sensor histidine kinase
hpk3	Complement (89872 - 91275)	two-component sensor histidine kinase
yesM	86651 - 88363	two-component sensor histidine kinase
lamC	Complement (66869 - 68128)	histidine kinase
plnB	Complement (78070 - 79242)	histidine kinase
	36326 - 36787	sensor histidine kinase
hpk1	Complement (41682 - 43556)	PAS domain-containing sensor histidine kinase
hpk7	Complement (4163 - 5686)	signal transduction histidine kinase

Nine genes predicted to encode bacteriocin immunity proteins were identified in the draft genome (Table 5). Bacteriocin producers typically express immunity proteins to protect themselves from their own bacteriocins and prevent self-toxicity. Three secretion genes were found in the genome of *L. plantarum* IYO1511, which suggests increased bacteriocin production as a strategy for improving bactericidal and bacteriostatic effects against pathogenic bacteria (Table 5).

Table 5. Identity of bacteriocin immunity protein and secretion protein found in *L. plantarum* IYO1511

Gene	Location	Putative Protein Encode
	10856 - 11212	bacteriocin immunity protein
plnI	75397 - 76170	bacteriocin immunity protein
plnP	Complement (80613 - 81359)	bacteriocin immunity protein
plnM	Complement (83001 - 83201)	bacteriocin immunity protein
plnL	84431 - 85099	bacteriocin immunity protein
	68651 - 68932	bacteriocin immunity protein
	10194 - 10493	bacteriocin immunity protein
	38165 - 38422	bacteriocin immunity protein
	2895 - 3224	bacteriocin immunity protein
gspE	34706 - 35680	secretion protein E
	35619 - 36665	secretion protein F
	37011 - 37481	type II secretion protein

Plantaricin are bioactive bacterial peptides or proteins produced by *L. plantarum* strains that exert antimicrobial effects against other bacteria. The genome of *L. plantarum* IYO1511 contains several genes that are predicted to be associated with plantaricin production (Fig. 3a): *plnW* (*Locus_18120*), *plnV* (*Locus_18130*), *plnU* (*Locus_18140*), *plnH* (*Locus_18160*), *plnG* (*Locus_18170*), *plnE* (*Locus_18180*), *plnF* (*Locus_18190*), *plnI*

(*Locus_18200*), *plnD* (*Locus_18210*), *plnC* (*Locus_18220*), *plnB* (*Locus_18230*), *plnA* (*Locus_18250*), *plnQ* (*Locus_18260*), *plnP* (*Locus_18270*), *plnN* (*Locus_18300*), *plnM* (*Locus_18310*), *plnJ* (*Locus_18320*), *plnK* (*Locus_18330*), and *plnL* (*Locus_18340*) (Table 6).

Table 6. Identity of putative proteins found in the bacteriocin *pln* gene of *L. plantarum* IYO1511

Gene	Location	Putative Protein Encode
plnW	Complement (68074 - 68760)	membrane protein
plnV	Complement (68854 - 69534)	membrane protein
plnU	Complement (69621 - 70289)	membrane protein
plnH	Complement (71136 - 72512)	bacteriocin ABC transporter
plnG	Complement (72528 - 74678)	bacteriocin cleavage/export ABC transporter
plnE	74945 – 75115	bacteriocin
plnF	75140 – 75298	bacteriocin
plnI	75397 – 76170	bacteriocin immunity protein
plnD	Complement (76464 - 77207)	DNA-binding response regulator
plnC	Complement (77326 - 78069)	DNA-binding response regulator
plnB	Complement (78070 - 79242)	histidine kinase
plnA	Complement (79589 - 79735)	bacteriocin plantaricin-A
plnQ	Complement (80071 - 80259)	hypothetical protein
plnP	Complement (80613 - 81359)	bacteriocin immunity protein
plnN	Complement (82706 - 82873)	bacteriocin

plnM	Complement (83001 - 83201)	bacteriocin immunity protein
plnJ	84069 – 84230	bacteriocin
plnK	84261 – 84434	bacteriocin
plnL	84431 – 85099	bacteriocin immunity protein

The genomes of *L. plantarum* IYO1511 and *L. plantarum* WCFS1 were compared (Fig. 3). We found that *plnI* was present in the genome of *L. plantarum* IYO1511 but not in the genome of *L. plantarum* WCFS1. *PlnS*, *plnT*, and *plnO* were present in the genome of *L. plantarum* WCFS1 but were not found in the genome of *L. plantarum* IYO1511. This shows that *L. plantarum* IYO1511 is a new strain in post-fermented tea that is different from other previously reported strains.

2.3.3.3 Antibacterial Activity of *L. plantarum* in Post-fermented Tea

The plantaricin genes *plnA*, *plnEF*, and *plnNJK* were found in *L. plantarum.* Antibacterial activity was tested to determine the functional effects, i.e., bactericidal and bacteriostatic effects, of *plnA*, *plnEF*, and *plnNJK* expression. Antibacterial properties were assessed by the presence of a clear halo surrounding bacterial cell extract from cells harvested at the beginning of stationary phase. Isolates with clear zones were considered positive. The plantaricin genes were expected to be expressed and, through the processes of transcription and translation [20], to produce bacteriocin, which is a bio-preservative of post-fermented tea.

Table 7. Inhibitory effects of *L. plantarum* isolated from post-fermented tea

Indicator Bacteria	Positive Control (mm)	*L. plantarum* IYO1501 (mm)	*L. plantarum* IYO1511 (mm)
E. coli K12 JM109	12	5.5	9.25
S. aureus JCM 20624	13.5	9.25	8.25
B. subtilis	10.75	8.25	10.25

Cell-free supernatant of *L. plantarum* strain IYO1501 and IYO1511 inhibited the growth of indicator bacteria (Table 7). Thus, it can be concluded that *L. plantarum* strains express *plnA*, *plnEF*, and *plnNJK* have bactericidal and bacteriostatic functions.

2.3.3.4 Plantaricin Plays a Role in the Fermentation Process

The *L. plantarum* genes *plnA*, *plnEF*, and *plnNJK* exert antibacterial activities, and these functions may affect the fermentation process. Genes associated with bacteriocin production were identified to confirm the presence of plantaricin genes in total DNA isolated from fermented tea, as these gene products may affect the fermentation process. Eight PCR primers specific to plantaricin genes were used to amplify these genes in each DNA sample from tea. *plnA*, *plnEF*, and *plnNJK* gene fragments were detected in samples collected after both primary and secondary fermentation, whereas *plnS*, *plnC8α*, *plnC8β*, *pln423A*, and *plnZJS* were not observed by PCR (Table 8).

Table 8. Identification of genes associated with bacteriocin production in fermented tea using specific primers

Producer	Product	*plnA*	*plnEF*	*plnS*	*plnNJK*	*plnC8α*	*plnC8β*	*pln423A*	*plnZJS*
A	Before Fermentation	-	-	-	-	-	-	-	-
	Primary Fermentation	++	+++	-	+++	-	-	-	-
	Secondary Fermentation	+++	++	-	+++	-	-	-	-
B	Before Fermentation	-	-	-	-	-	-	-	-
	Primary Fermentation	+++	+++	-	+++	-	-	-	-
	Secondary Fermentation	+++	+++	-	+++	-	-	-	-
C	Before Fermentation	-	-	-	**ND**	-	-	-	-
	Primary Fermentation	+	+	-	**ND**	-	-	-	-
	Secondary Fermentation	+	+	-	**ND**	-	-	-	-

ND: Not detected

Samples obtained from manufacturers A and B were harvested three times; (+++) indicates that all three samples contained plantaricin, (++) indicates that two samples contained plantaricin, (+) indicates that one sample contained plantaricin, and (-) indicates that no plantaricin was detected. Samples from manufacturer C were harvested once; (+) indicates that samples contained plantaricin, (-) indicates that no plantaricin was detected.

DNA fragments of *plnA*, *plnEF*, and *plnNJK*, with sizes of approximately 550, 550, and 1,500 bp, respectively, were amplified from total DNA of each tea sample. We estimated the number of genes present during the fermentation process, and the results showed that plantaricin genes increased after both primary and secondary fermentation. Thus, it can be concluded that plantaricins play a role during the fermentation process.

2.3.4 Discussion

In this study, lactic acid bacteria were found to play a role in the fermentation of Ishizuchi-kurocha tea. Horie (2019) reported that *Lactobacillus* spp. could not be isolated from raw Ishizuchi-kurocha tea leaves; however, *Lactobacillus* spp. could be isolated after aerobic and anaerobic fermentation. After anaerobic fermentation of Ishizuchi-kurocha tea, *Lactobacillus* was the only isolated genus of lactic acid bacteria, and *L. plantarum* was the most frequently isolated species after aerobic and anaerobic fermentation [53]. In the current study, *L. plantarum* was used because of its ability to survive under both aerobic and anaerobic conditions. Two *L. plantarum* strains were isolated from Ishizuchi-kurocha tea leaves, IYO1501 and IYO1511 [52]. The pH value of the tea leaf extract from Ishizuchi-kurocha ranged from 3.8 to 4.4, which is slightly lower than that of the Awa-bancha and Goishi-cha extracts [53]. Horie (2019) reported that Ishizuchi-kurocha is more acidic than other post-fermented teas. The acidity produced by *L. plantarum* is likely what makes this species the dominant lactic acid bacteria in the final product [53]. Under anaerobic, low pH conditions, *L. plantarum* grew well and exerted bactericidal and bacteriostatic effects. However, the role of plantaricin in Ishizuchi-kurocha tea processing has not been reported thus far.

L. plantarum was predicted to possess plantaricin genes both in the chromosome and on plasmids, and this was tested throughout the fermentation process by PCR using eight specific primers. The results showed that *plnA* and *plnEF* were present on plasmids of *L. plantarum*, and *plnNJK* was present on the *L. plantarum* IYO1511 chromosome. In contrast, *plnA* was not detected in the total DNA of *L. plantarum* IYO1501. However, *plnA* was detected on a plasmid extract from *L. plantarum* IYO1501 prepared using an alkaline lysis method. Plasmid stability and copy number may differ between strains. Plantaricin genes may be transferred by horizontal gene transfer to bacteria that have compatible plasmids [64]. Bacteria that can receive these transferred genes are assumed to possess compatible plasmids in order to

adopt the plantaricin genes. However, if the plasmids are incompatible, the gene will be released from the cell, and the cell must find an alternative mechanism to survive and inhabit certain environments without the aid of such transferred plasmids. The transfer pathway depends on replication mechanisms within the cell [64]. These mechanisms may help during fermentation to counteract any pathogenic bacteria that may survive the fermentation process. These genes may be transferred to closely related bacteria that possess compatible plasmids, which facilitates accepting plantaricin genes. Species that possess an adequate genetic structure will survive.

The draft genome of *L. plantarum* IYO1511 contained genes encoding bacteriocin immunity proteins, which are essential for bacteria, as these proteins can prevent self-toxicity [59][60]. The *L. plantarum* IYO1511 draft genome showed that the *plnEF* secretion gene, which likely produces bacteriocin, suggesting that *plnEF* expression may be more important for the bactericidal and bacteriostatic effects observed against related bacteria species than other plantaricin genes [50]. However, this hypothesis requires further studies to determine which plantaricin gene is particularly important.

L. plantarum strains express *plnA*, *plnEF*, and *plnNJK* showed bactericidal and bacteriostatic effects. The plantaricin genes were expected to be expressed and, through the processes of transcription and translation [65], to produce bacteriocin, which is a bio-preservative of post-fermented tea. Antimicrobial activity assays showed that *L. plantarum* inhibited the growth of three indicator strains, *S. aureus* JCM 20624, *E. coli* K12 *JM109*, and *B. subtilis*. Several reports showed that plantaricin has antimicrobial effects, including studies on *plnA* and *plnEF* in *L. plantarum C11*, which was isolated from traditional Motal cheese, it showed activity against *S. aureus* ATCC 25923, *E. coli* ATCC 25922, and *Listeria innocua* ATCC 33090[1]. *plnEF* and *plnNJK* encode dipeptide bacteriocins that are 10^3-fold more active

when they are combined with their complementary peptide than when they occur individually [50][21].

L. plantarum expresses *plnA, plnEF* and *plnNJK* are known to exerts antimicrobial effects, and their functions were examined during fermentation. The results showed *plnA*, *plnEF*, and *plnNJK* accumulated during primary and secondary fermentation. Plantaricin genes likely play a role during fermentation; however, the acidity of the tea is also bactericidal and bacteriostatic [53]. Horie (2019) reported that acidity increases during fermentation. Plantaricin is not only bactericidal and bacteriostatic, but may also contribute the production of fermented foods and may thus have contributed to food production. The present study estimated the amount of these genes during fermentation, and plantaricin genes were found to increase during primary fermentation. This showed that plantaricin plays a key role during fermentation by functioning to inhibit pathogenic bacteria so that *L. plantarum* can survive under aerobic conditions.

2.3.5 Conclusion

The examined plantaricin genes were expected to be expressed and to produce bacteriocin, which functions as a bio-preservative in post-fermented tea. Plantaricin affects the fermentation process. Although the antimicrobial effects of plantaricin are relatively well understood, the functions of *plnA*, *plnEF*, and *plnNJK* expression during tea fermentation have only been examined in the processed product. Our results showed that plantaricin genes were present on the plasmids and chromosomes of *L. plantarum* IYO1501 and *L. plantarum* IYO1511 and play an important role in successful tea processing.

The term “fermented tea” is used for English tea and oolong tea. However, these teas are not fermented by microorganisms. Therefore, a specific term referring to microbially fermented tea, such as post-fermented tea, should be used. However, the term “post-fermented tea” does not necessarily reflect the process of tea production. Therefore, we suggest the term “brewing tea” to refer to lactic acid bacteria-fermented tea.

2.4 Application of Plantaricin Produced by *Lactobacillus plantarum* IYO1511 on Post-fermented of Coarse Tea (Bancha) using the Artificially Fermented Method

2.4.1 Introduction

Tea is one of the most popular beverages worldwide and also one of the important commodities in Japan. Japan has diverse tea types, including non-fermented tea, semi-fermented teas, fully fermented tea, and post-fermented tea [54]. Coarse tea (bancha) is a mature tea plant and generally, it is a lower quality tea compared to tender shoots fresh tea. Coarse tea (bancha) is regarded as a worthless crop product [61] and harvested between summer and autumn. One of the techniques to storing and making high price value of tea commodities is fermenting them [62] and fermented tea is popular in Japan.

In this research, the coarse tea (bancha) was obtained from Ibigawa town, Gifu Prefecture Japan. We tried to ferment the coarse tea (bancha) as good as commercial tea that using tender shoots fresh tea and develop the fermentation process of coarse tea into an artificially fermented method. The commercial post-fermented tea such as Ishizuchi-kurocha has abundant of lactic acid and made it has characteristic in acidic taste. Through this acidic taste, we make it as a standard to develop the taste of tea. Generally, to make post-fermented tea takes two weeks until one month for the fermentation process [53]. However, in the previous research, we successfully cut the fermentation time and develop a new model system using *Aspergillus luchuensis mut. Kawachii* as the main fungi. The use of *Aspergillus luchuensis mut. Kawachii* showed the highest lactic acid concentration than other fungi and commercial tea in the previous research [66].

In other previous research showed that *L. plantarum* IYO1511 acted as bacteriostatic and bactericidal and produced plantaricin as an antimicrobial substance to increase the food-safety. The fermentation was carried out 4 days aerobically and anaerobically respectively

according to previous research [66]. In this research, we focused to confirm the plantaricin, physicochemical and microbial analysis on the artificially fermented method as bio-preservative during fermentation.

2.4.2 Materials and Methods

2.4.2.1 Screening *L. plantarum* strains

Three kinds of *L. plantarum* strains isolated from VCO and secondary fermentation of Ishizuchi-kurocha, *L. plantarum* strain COY 2906, IYO1501 and IYO1511 [53] & [52], were screened for further study. These *L. plantarum* strains produced lactic acid and the highest production will be considered as the main inoculum for the next research. Lactic acid was determined with a protocol based on *Spectrophotometric Determination of Lactic Acid* [41]. Lactic Acid (Wako Pure Chemical Industries, Japan) was used as a standard curve with equation calibration curve y = 0.5969x + 0.6972, the correlation coefficient is 0.979 and determined using Spectrophotometry (Molecular Device SpectraMax M5, US). During the manufacturing process, initial and final weights are weighed using a balancer (Mettler Teledo PB3002-S, Columbus).

2.4.2.2 RNA Extraction

One milliliter of culture *L. plantarum* strains were centrifuged at 14,000 × *g* for 5 min. The supernatants were removed, and the pellets were resuspended in 300 µL (50mM Sodium acetate, 10mM EDTA). The solution was incubated at 65°C for 5min and centrifuged at 14,000 × *g* for 5min. The upper side solution was transferred to a new tube and added with 300 µL phenol: chloroform (1: 1) and centrifuged at 14,000 × *g* for 5min. The upper side solution was transferred to a new tube and added with 300 µL chloroform (Wako Pure Chemical, Japan), the addition of chloroform was done 2 times. The supernatant was centrifuged at 14,000 × *g* for 5min, the upper side solution was transferred to a new tube and added 10µL 3M sodium acetate (pH 5.3) and 257 µL 99.5% ethanol (Wako Pure Chemical, Japan). The solution was kept for 1 night and centrifuged at 14,000 × *g* at 4 ˚C for 30min. The supernatant was removed and resuspend with 500 µL 70% ethanol and centrifuged at 14,000 × *g* at 4 ˚C 1min. The supernatant

was removed and dried ± 2h at room temperature. The pellet was resuspended in 20 µL nuclease-free deionized water.

2.4.2.3 Reverse transcription and RT qPCR on *L. plantarum* strains and Post-fermented Tea Artificially Fermented Method

cDNA was synthesized using TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, USA). The plantaricin genes, *plnA, plnEF, plnN, plnJ,* and *plnk* of *L. plantarum* was determined by Real Time qPCR (ABI Step One Plus, Thermo Fisher Scientific Inc.). Real Time qPCR was performed with 10 µL final volume containing 1 µL of cDNA template, 1 µL of each primer at a concentration of 0.5 µM (Table 3), 2 µL of RNAse free-water, and 5 µL of Power SYBR® Green PCR Master Mix (Applied Biosystems, Thermo Fisher Scientific, UK) with an initial step at 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s, 60 °C for 1 min and 72 °C for 30 s. *16S rRNA* was selected as housekeeping genes on the basis of the available literature. Results were analyzed using the comparative critical threshold method (ΔCT) in which the amount of target RNA was adjusted to a reference (internal target RNA) [44]. They were taken in triplicate for each gene.

Table 3. Primer sequences and respective amplicon sizes used for the in vitro and in situ gene expression assay

Target Genes	Sequence	Amplicon size	Reference
Housekeeping			
16S rRNA_F	GATGCATAGCCGACCTGAGA	114	[38]
16S rRNA_R	CTCCGTCAGACTTTCGTCCA		
Plantaricins			
plnA_F	AAAATTCAAATTAAAGGTATGAAGCAA	108	
plnA_R	CCCCATCTGCAAAGAATACG		
plnEF_F	GTTTTAATCGGGGCGGTTAT	85	
plnEF_R	ATACCACGAATGCCTGCAAC		
plnN_F	GCCGGGTTAGGTATCGAAAT	102	[44]
plnN_R	TCCCAGCAATGTAAGGCTCT		
plnJ_F	TAAGTTGAACGGGGTTGTTG	102	
plnJ_R	TAACGACGGATTGCTCTGC		
plnK_F	TTCTGGTAACCGTCGGAGTC	97	
plnK_R	ATCCCTTGAACCACCAAGC		

2.4.2.5 Microorganism and Inoculum Preparation

Aspergillus luchuensis mut. Kawachii was obtain from Kojiya Sanzaemon (Toyohashi, Japan). *L. plantarum* strain IYO1511 previously isolated from secondary fermentation of post-fermented tea from Ishizuchi-kurocha [53] [52]. Throughout this study due to its competitive potential, lactic acid and plantaricin production. *L. plantarum* strain IYO1511 was preincubated in MRS broth (Becton, Dickinson and Company, USA) at 37°C for 24 h. Stock cultures were stored at −80°C in MRS broth containing 20% glycerol. *L. plantarum* culture (9 log CFU/mL) was used to inoculate the fermented tea in MRS broth.

2.4.2.6 Sampling and Post-fermented Tea Preparation

The dry tea was obtained from Ibigawa town, Gifu Prefecture Japan and mixed with water with ratio 1: 1.5 and kept for 30 min at 4ºC. The procedure to make post-fermented tea followed according to the directions of [66] and [53]. The tea leaf was steamed in autoclave for 1h, temperature was controlled at 85 - 90°C. Thirty grams steamed tea leaves were packed in plastic boxes with small holes in the bottom. Added 1‰ *Aspergillus luchuensis mut. Kawachii,* then mixed fully. The preparation process was done with two models. First artificially fermented model, *Aspergillus luchuensis mut. Kawachii* and *L. plantarum* strain IYO1511 were adding them together (T) and fermented it in aerobic and anaerobic condition for 4 days, respectively. Second artificially fermented model, *Aspergillus luchuensis mut. Kawachii* was used in the first fermentation (aerobic condition) for 4 days and after finished, leaves were mixed with *L. plantarum* IYO1511 for 4 days (R), respectively. The fermentation was done at 37°C in incubator and the culture was used 50 µL/30 g tea of *L. plantarum* IYO1511. The tea leaves were harvested at each step of the fermentation process. The samples were harvested in duplicate for each fermentation process.

2.4.2.7 Physico-Chemical and Microbiological Analyses on Post-fermented Tea

Monitoring of the fermentation was performed by measuring pH value, lactic acid concentration and mass lost in the samples. Ten milliliters of hot water were added to 500 mg of tea leaves in the test tube and marinated for 30 min. The pH of the solution was measured using a pH meter (AS ONE AS800, Japan). Lactic acid was determinated with a protocol based on *Spectrophotometric Determination of Lactic Acid* [41]. Microbiological analyses were performed in the samples, lactic acid bacteria (LAB), viable bacteria and yeasts were performed according to Horie's procedure [53]. Tea leaves were collected at each step of the fermentation process. All analyses were performed in duplicate and the average values are presented.

2.4.2.8 Antibacterial Effects of Post-fermented tea using Artificially Fermented Method

The antibacterial effects of tea was estimated using the disk diffusion method [22], and the result was evaluated based on the presence of a clear halo surrounding the disk. One gram of each sample tea was dissolved in 4mL PBS (Takara Bio, Japan) and the supernatant was centrifuged and used for the assay. *Escherichia coli* K12 JM109, *Bacillus subtilis*, and *Staphylococcus aureus* JCM 20624, which were used as the indicator bacteria, were grown in Luria Bertani broth (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) at 30°C for 12 h. The disks were placed on Mueller-Hinton agar (Becton, Dickinson and Company) and were impregnated with 100 µL of cell-free supernatant of tea. Plates were incubated at 30°C for 24 h to assess antibacterial activity as halo formation (i.e., the zone of growth inhibition). Isolates that produced clear zones, indicating growth inhibition, with a diameter >1 mm were considered positive.

2.4.3 Results

2.4.3.1 *L. plantarum* IYO1511 has Higher Lactic Acid Production than other strains

L. plantarum strains were screening to determine the use of LAB for next further research. As high lactic acid concentration is the main characteristic of post-fermented tea, lactic acid concentration was used for screening *L. plantarum* strains. Figure 4 showed that lactic acid produced by IYO1511 is higher than other strains. For the next step fermentation, IYO1511 will be used as main lactic acid bacteria.

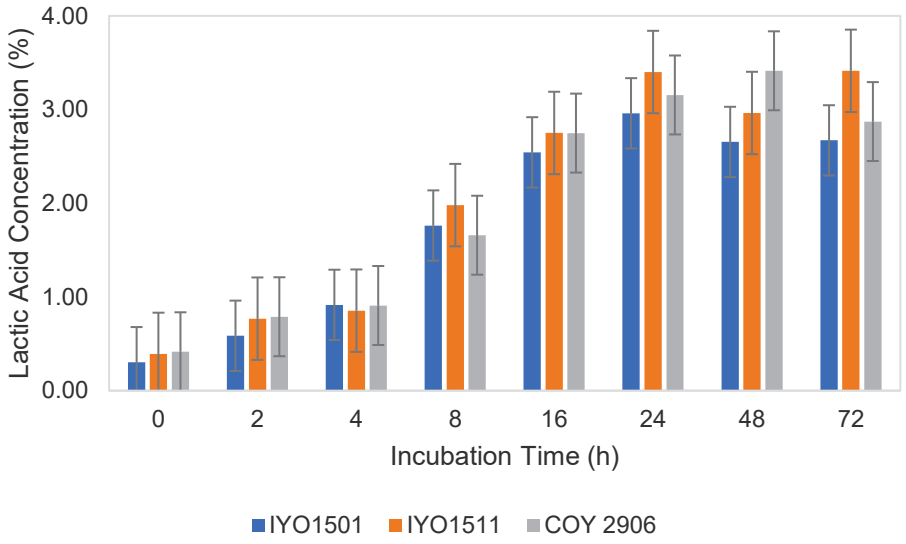

Fig. 4 Lactic acid production by *L. plantarum* strains

2.4.3.2 Relative Expression of Plantaricin on *L. plantarum* IYO1511 is Higher than other strains

The focused study on *L. plantarum* strains were *plnA, plnEF, plnN, plnJ,* and *plnK*. The relative expression (RE) level of each gene was calculated to determine the genes responsible for bioactivity. Fig. 5 presents the expression of *plnA, plnEF, plnN, plnJ,* and *plnK* genes during the growth of *L. plantarum* strains in MRS. *L. plantarum* IYO1501 showed did not give any expression on *plnA* and *plnK.* Relative expression of *plnEF, plnN,* and *plnJ* of *L. plantarum* IYO1511 showed higher than *L. plantarum* other strains. *L. plantarum* COY 2906 showed did

not express *plnK*. For the next step fermentation, IYO1511 will be used as main lactic acid bacteria.

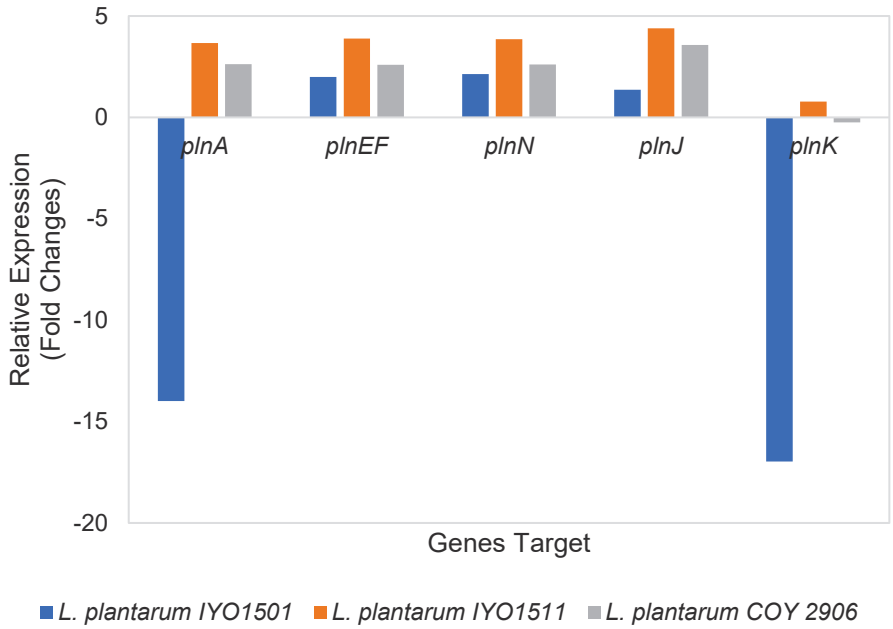

Fig. 5 Relative expression (RE) levels of the *plnA, plnEF, plnN, plnJ,* and *pln K* genes of *L. plantarum* strains cultured on MRS

2.4.3.3 Physico-chemical and microbiological changes during fermentation

Table 11 showed the physico-chemical and microbiological changes during fermentation. Li (2020) explained that the best fermentation time is 8 days, which is 4 days for first and secondary fermentation with aerobic and anaerobic fermentation, respectively. During fermentation, the pH of fermentation was changed from 5.6 to 4.4 and 5.8 to 4.2 for 1st model (T) and 2nd model (R), respectively. The pH of commercial tea from Ishizuchi-kurocha changed from 5.7 to 4 during fermentation. The pH of commercial tea and these model fermentation methods are not significantly different, so we can be concluded that the acidic level of these models has been successfully made.

The LAB was found at every single step in the 1st artificially fermented model systems (T) during fermentation. The number of LAB colonies was increasing, especially in anaerobic

conditions. It is different from the 2nd artificially fermented model systems (R) that did not have any LAB in aerobic conditions. However, in the last of the product, the number of LAB in the 1st (T) and 2nd (R) artificially fermented model were found much higher than commercial tea. In table 11 for the total colony of viable bacteria, we found that the 2nd artificially fermented model systems (R) is better than the 1st model system (T), it showed that the number of colonies on the 1st artificially fermented model system (T) is higher than the 2nd artificially fermented model systems (R). However, we found that the colony number of viable bacteria in the model systems that we made was the same (1st model systems) and lower (2nd model systems) with/than commercial tea.

The colony number of fungi is increasing during aerobic fermentation and decreasing during anaerobic fermentation for both of fermentation models (Table 11). The colonies number of fungi in the last fermentation were $11x10^{1}$ and $3x10^{1}$ on the 1st model system (T) and 2nd artificially fermented model systems (R), respectively. The colony number of fungi in the 1st and 2nd artificially fermented model systems showed lower than commercial tea. In addition, the concentration of lactic acid during fermentation was fluctuating. However, it can be concluded that the concentration of lactic acid was increasing during fermentation. Table 11 showed that lactic acid concentration increased become 0,4% and 0.46% for 1st (T) and 2nd (R) artificially fermented model systems, respectively. The lactic acid of commercial tea in the last fermentation was 0.14%. This means the lactic acid concentration in the 1st and 2nd artificially fermented model system was higher than commercial tea. During first fermentation, there were mass loss content in tea for 1st and 2nd artificially fermented model systems. However, the mass lost content did not occur in the second fermentation by the anaerobic condition.

1

Table 11. Physico-chemical and microbiological changes during fermentation

Condition	Day	pH	Total Colony			Lactic Acid Concentration	Mass lost (%)	Reference
			LAB	Viable Bacteria	Fungi			
1st Model								In this study
	0	5.613	$53x10^3$	$22x10^3$	$21x10^3$	0.003		
aerobic	1	4.354	$43x10^4$	$8x10^1$	$38x10^4$	0.0806 ± 0.079	23.88	
	2	5.402	$2x10^5$	$7x10^4$	$22x10^6$	0.4285 ± 0.033	44.26	
	3	5.729	$7x10^6$	$9x10^5$	$9x10^6$	0.4487 ± 0.202	54.47	
	4	5.808	$1x10^6$	$2x10^5$	$16x10^6$	0.5107 ± 0.042	55.07	
anaerobic	5	4.835	$54x10^{10}$	$51x10^7$	$16x10^6$	0.5398 ± 0.056	0	
	6	4.565	$16x10^{12}$	$5x10^8$	$6x10^5$	0.6503 ± 0.009	0	
	7	4.398	$9x10^{13}$	$5x10^9$	$6x10^2$	0.5465 ± 0.040	0	
	8	4.417	$41x10^{13}$	$19x10^7$	$11x10^1$	0.4012 ± 0.045	0	
2nd Model								In this study
aerobic	0	5.751	0		$1x10^2$	0		
	1	4.387	0		$6x10^3$	0.1557	27.73	
	2	5.358	0		$56x10^5$	0.2271 ± 0.041	53.64	
	3	5.384	0		$7x10^6$	0.3399 ± 0.035	64.41	
	4	5.536	0		$8x10^6$	0.3596 ± 0.005	65.57	

anaerobic	5	4.43	$21x10^6$	$14x10^7$	$2x10^3$	0.321 ± 0.041	0	
	6	4.263	$3x10^7$	$37x10^6$	$2x10^2$	0.4041 ± 0.063	0	
	7	4.163	$15x10^7$	$9x10^6$	$2x10^2$	0.4556 ± 0.087	0	
	8	4.158	$19x10^9$	$13x10^4$	$3x10^1$	0.4604 ± 0.012	0	
Ishizuchi-kurocha								
Before Fermentation		5.645	10^2-10^3	10^3		0		In this study, [53] & [54]
First Fermentation		5.175	10^7	10^7	10^6-10^8	0.057		
Secondary Fermentation		3.995	10^8	10^7-10^8	10^4-10^8	0.14		

3

2.4.3.4 Post-fermented Tea has Antimicrobial Activities

Antimicrobial activity was checked to determine the role of plantaricin in the fermentation process as bio-preservative. It reported before that *L. plantarum* IYO1511 encoded plantaricin has antimicrobial activity. Table 12 showed that 1st and 2nd artificially fermented model system did not give any inhibition on aerobic condition, except on day 4 of 1st artificially fermented model system with the indicator of *S. aureus* JCM 20624. On anaerobic condition 1st and 2nd artificially fermented model system gave inhibition to indicator bacteria, except on day 5 on *E. coli* K12 JM109 and *B. subtilis*. Plantaricin has role as bactericidal and bacteriostatic in this fermentation process. However, the halo zone that showed is much smaller than control positive.

Table 12. Antimicrobial activities during fermentation of coarse tea (bancha)

Condition	Day	Indicator Bacteria		
		E. coli K12 JM109	*B. subtilis*	*S. aureus* JCM 20624
Control Positive	1	++++	++++	+++
1st Model				
aerobic	1	-	-	-
	2	-	-	-
	3	-	-	-
	4	-	-	+
anaerobic	5	-	-	+
	6	+	+	+
	7	+	+	+
	8	+	+	+
2nd Model				
aerobic	1	-	-	-
	2	-	-	-
	3	-	-	-
	4	-	-	-
anaerobic	5	-	-	+
	6	+	+	+
	7	+	+	-
	8	+	+	+

Note: ++++ : ≥ 9.1 mm; +++: 6.1 – 9.0 ; ++: 3.1 – 6.0 mm; +: 0 – 3.0 mm

2.4.4 Discussion

The *L. plantarum* strains were screened by lactic acid production and relative expression of plantaricin, the highest production will be considered as the main inoculum for the next research. The *L. plantarum* IYO1511 showed the highest lactic acid production and relative expression of plantaricin. For further study, we used *L. plantarum* IYO1511 as main LAB to artificially fermented method. We used two kinds of artificially fermented models to make post-fermented from coarse tea (bancha). During fermentation, *Aspergillus luchuensis mut. Kawachii* and *L. plantarum* strain IYO1511 were used as the main microorganisms. The result of Li (2020) on artificially fermented method showed that *Aspergillus luchuensis mut. Kawachii* produced higher lactic acid than other koji fungi and commercial tea. As high lactic acid concentration showed the main characteristic of post-fermented tea, we chose *Aspergillus luchuensis mut. Kawachii* as the main fungus.

The pH value of the tea leaf extract from Ishizuchi-kurocha ranged from 3.8 to 4.4, which is slightly lower than that of the Awa-bancha and Goishi-cha extracts [53]. Horie (2019) reported that Ishizuchi-kurocha is more acidic than other post-fermented teas. The acidity produced by *L. plantarum* is likely what makes this species the dominant lactic acid bacteria in the final product [53]. Under anaerobic, low pH conditions, *L. plantarum* grew well and exerted bactericidal and bacteriostatic effects. Li (2020) explained that the best fermentation time is 8 days, which is 4 days for first and secondary fermentation with aerobic and anaerobic fermentation, respectively. We found that the pH of commercial tea and theses models fermentation methods are not significantly different, so it can be concluded that the acidic level of these models has been successfully made. The number of LAB colonies was increasing, especially in anaerobic conditions. *L. plantarum* IYO1511 is an anaerobic bacterium that has suitable conditions for growth [53]. However, the 2nd artificially fermented model systems (R) did not show LAB during aerobic condition. In the last of the product, the number of LAB in

the 1st (T) and 2nd (R) artificially fermented model were found much higher than commercial tea. This condition has the advantage to make tea products safer than commercial tea. On the artificially fermented tea, we found that the colony number of viable bacteria in the model systems that we made was the same (1st model systems) and lower (2nd model systems) with/than commercial tea.

The colony number of fungi in the 1st and 2nd artificially fermented model systems were lower than commercial tea. On the commercial tea, *Aspergillus niger* plays a role of the first step fermentation of post-fermented tea [54]. *A. niger* may produce mycotoxins that made the post-fermented tea product not safety. Li (2020) confirmed *A. niger* using specific primer in these artificially fermented methods, they found that *A. niger* could not be detected in all artificial fermented tea samples. The decreasing of *A. niger* could make tea products safer and adding of industrial koji fungus efficiently inhibited the growth of *A. niger* [66].

The concentration of lactic acid was increasing during fermentation and the lactic acid concentration in the 1st and 2nd artificially fermented model system was higher than commercial tea. The benefit in this case, the number of lactic acids will make strongly acidic taste characteristics, make the environment suitable for *L. plantarum* IYO1511 and make tea safer to consumed than commercial tea. During first fermentation, there was a loss of mass content in tea for 1st and 2nd artificially fermented model systems. However, the mass lost content did not occur in the second fermentation by the anaerobic condition. Artificially fermented method on the 1st and 2nd models did not give any inhibition on aerobic condition, except on day 4 of 1st artificially fermented model system with the indicator of *S. aureus* JCM 20624. Syaputri (2020) reported that this *S. aureus* JCM 20624 is more sensitive than other indicator bacteria. On anaerobic condition 1st and 2nd artificially fermented model system gave inhibition to indicator bacteria, except on day 5 on *E. coli* K12 JM109 and *B. subtilis*. However, in the last of product, these models gave inhibition to indicator bacteria. Plantaricin has role as

bactericidal and bacteriostatic in this fermentation process as bio preservation. From this study, we success to make post-fermented tea with artificial fermented method and these showed that plantaricin plays a key role during fermentation by functioning to inhibit pathogenic bacteria so that *L. plantarum* can survive under aerobic conditions.

3. CONCLUSION

Lactobacillus plantarum is a lactic acid bacterium found in nutrient-rich environments such as plants, meat, fish, and dairy products. *L. plantarum* produces organic acids, fatty acids, ammonia, hydrogen peroxide, diacetyl, and bacteriocin as well as other substances to grow and survive in its environment. The bacteriocin produced by *L. plantarum* is known as plantaricin and is generally reported as a class II bacteriocin, a very broad class with a variety of bactericidal/bacteriostatic mechanisms. Class II bacteriocins are small peptide (< 10 kDa), heat-stable molecules with isoelectric points varying from 8.3 to 10.0. Differences in structural amino acids result in different characteristics of plantaricins, such as resistance to pH and temperature and antimicrobial activity. Differences are also influenced by the location of the plantaricin-coding genes. In *L. plantarum,* these are located in operon clusters, which may be located on chromosomes, plasmids, or transposons. Commonly, plantaricin disrupts cell wall integrity and inhibits protein or nucleic acid synthesis. It has been reported that the bacterial membrane is the target of bacteriocins.

In this research, we tried to isolate *L. plantarum* from many samples; soil, Virgin Coconut Oil (VCO) and post-fermented tea of Ishizuchi-kurocha. We found that three of six *L. plantarum* isolates from healthy soil and one of four *L. plantarum* isolates from soil that has black rot disease in brassica plant showed the presence of *plnA.* We continued the research using other samples, Virgin Coconut Oil (VCO). *L. plantarum* COY 2906 isolated from VCO was showing great ability to inhibit indicator bacteria by producing plantaricin. Under saline stress and alkali conditions, *L. plantarum* COY 2906 can inhibit *E. coli* K12 JM10, *B. subtilis* and *S. aureus* JCM 20624. The copy number of *plnA, plnEF, plnN, plnJ,* and *plnK* genes, associated with plantaricin, were not significantly changed under saline condition. However, the multiplication of plantaricin plasmid genes reduced under alkali condition.

We continued the research using other samples, *L. plantarum* strains IYO1501 and IYO1511 were isolated from post-fermented Ishizuchi-kurocha tea. We found that plantaricin genes *plnA* and *plnEF* were detected on plasmids of *L. plantarum* IYO1501 and IYO1511, and *plnNJK* was detected in the *L. plantarum* IYO1511 chromosome. Regarding antimicrobial activity, the expression products of *plnA*, *plnEF*, and *plnNJK* exerted bactericidal and bacteriostatic effects on indicator bacteria. Using eight specific PCR primers, genes associated with bacteriocin production were examined throughout the fermentation process, i.e., before fermentation and after primary and secondary fermentation. we found that *plnA*, *plnEF*, and *plnNJK* were expressed during primary and secondary fermentation.

These researches showed that plantaricin plays an important role as bio-preservation. We tried to apply these plantaricins in new model system to make a new product, pot-fermented tea. In this research, the coarse tea (bancha) was obtained from Ibigawa town, Gifu Prefecture Japan. *Aspergillus luchuensis mut. Kawachii* was used as the main fungi and *L. plantarum* IYO1511 as the main lactic acid bacteria. We succeed to equal the pH of these model fermentation methods as commercial post-fermented tea, increased lactic acid colony number and lactic acid production, and decreased the colony number of viable bacteria and fungi. These artificially fermented models did not give any inhibition on the aerobic conditions. However, in the last of the product, these models gave inhibition to indicator bacteria during anaerobic condition by producing plantaricin. We success to make post-fermented tea with artificial fermented method and showed that plantaricin plays a key role during fermentation by functioning to inhibit pathogenic bacteria.

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