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Characterization of Plantaricin Genes and Lactic Acid Production by *Lactiplantibacillus plantarum* strains Isolated from Ishizuchi-Kurocha

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Abstract

Background and Objective: Ishizuchi-kurocha is a post-fermented tea that involved two main kinds of microorganisms, namely fungi and lactic acid bacteria, which are primary and secondary fermentation, respectively. Therefore, this research aimed to confirm the role of *Lactiplantibacillus plantarum* during secondary fermentation of Ishizuchi-kurocha and the anti-bacterial effect due to lactic acid production and genes detection of plantaricin.

Material and Methods: Antimicrobial were estimated using well diffusion method. Lactic acid was determined with spectrophotometric method. Detection of plantaricin genes were confirmed by Real-Time qPCR. The genes were sequenced through DNA Sequencing Analytical service by the Division of Genomic Research, Gifu University using the Multi-capillary DNA Sequencer ABI Prism 3100/3130 Genetic Analyzer and the data analyzed by the CLC Sequence Viewer 8.0 and BioEdit 7.2. Statistical analysis was evaluated by one-way of variance followed Tukey's *post hoc* test using RStudio version 4.1.3.

Results and Conclusion: *L. plantarum* strain IYO1511 has higher antibacterial activities than strain IYO1501. In addition, *L. plantarum* strain IYO1511 produced higher lactic acid than strain IYO1501 and has plantaricin genes, *plnA*, *plnEF*, *plnN*, *plnJ* and *plnK*. However, *L. plantarum* strain IYO1501 only encoded *plnEF*, *plnN*, and *plnJ*. The plantaricin genes from the strains IYO1501 and IYO1511 were sequenced to identify the heterologous gene clusters of each species. It was discovered that *plnA*, *plnEF*, and *plnJ* of *L. plantarum* IYO1511 showed 100% similarity homology toward GenBank. The *plnN* of strain IYO1511 and *plnEF* of IYO1501 present extra base pairs inserted into the DNA. *L. plantarum* strains can be used as food preservative for artificial fermentation to control the safety and quality of the product of Ishizuchi-kurocha.The lactic acid and plantaricin were expected to inhibit pathogenically and spoilage bacteria to produce a unique acidic flavor as well as fragrance to Ishizuchi- kurocha.

Conflict of interest: The authors declare no conflict of interest.

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1. Introduction

Lactiplantibacillus plantarum, previously known as *Lactobacillus (L.) plantarum* [1], is a lactic acid bacterium (LAB) found in fish [2], meat [3], plant [4], and fermented food such as post-fermented tea [5]. There are different types of tea globally, including non-fermented, semi-, fully, and post-fermented tea. Ishizuchi-kurocha is one of the most special post-fermented tea in Saijo, Ehime, Japan, which is processed through a two-stage fermentation process, namely

primary fermentation (aerobic) and secondary fermentation (anaerobic) for 14 days for each stage. *Pichia kudriavzevii* and *Pichia manshurica* have a utility during primary fermentation in aerobic conditions [6], while *L. plantarum* plays a utility in the secondary fermentation of Ishizuchi-kurocha in anaerobic conditions [7]. Although Ishizuchi-kurocha has distinctive acidic tastes associated with secondary fermentation by *L. plantarum*, the biosafety and quality of the product during the process are difficult to control. The genus of *Lactobacillus, Klebsiella, Aerosakkonem*a, *Pseudomonas, Enterobacter, Bifidobacterium, Leuconostoc, Pseudocitrobacter, Weissella, Rhodoligotrophos, Methylobacterium,* and *Staphylococcus* were reported to detect from the fermented tea by Next Generation Sequence (NGS) [8].

L. plantarum can be used as a bio-preservative because it provides organic acids, fatty acids, ammonia, hydrogen peroxide, diacetyl, together with bacteriocin as bactericidal and bacteriostatic [9]. Lactic acid is the main organic acid that metabolizes pentose by homofermentative [10] and a bacteriocin is ribosomally synthesized peptides that are organized through genetic operons, which usually contained structural genes [11]. Meanwhile, protein-coding genes are involved in post-translational modifications and transport [12], which inhibit pathogenic and spoilage bacteria [13,14]. *L. plantarum* produces bacteriocin, namely plantaricin. Plantaricin E/F (plnEF) and lactic acid has successfully inhibited food pathogen, namely *Aeromonas hydrophila* by releasing lipopolysaccharide (LPS) and interact with the inner cell membrane of. *A. hydrophila* [15]. Furthermore, lactic acid and hydrogen peroxide (H_2O_2) synergistically enhanced bactericidal and generated hydroxyl radicals based on the intracellular iron ions which indicated DNA damage caused by cell death [16]. Lactic acid is delivered by the glycolysis pathway under anaerobic conditions from hexoses and pentoses on LAB metabolism pathways [17]. Due to the high production of lactic acid, Ishizuchi-kurocha has a unique sour taste and aroma [5].

Plantaricin are referred to as class II bacteriocins and divided to become class IIa, IIb, IIc, and IId [11]. Their amino acid is highly diverse, thus, to detect the amino acid is more efficient to design specific primer to amplify the plantaricin. The plantaricin gene is located at the operon clusters and can be exist on the chromosome, or as a plasmid that also occurs in transposons [18]. *L. plantarum* strain COY 2906 was reported to produce lactic acid, while *plnA, plnEF, plnN, plnJ,* and *plnK* exhibited antimicrobial activity under salinity and pH stress conditions. These bacteriocins have a specific way to inhibit and kill pathogenic bacteria. The common mechanism is by disrupting cell wall integrity and inhibiting protein or nucleic acid synthesis [19]. Therefore, it was assumed that *L. plantarum* has the function of a biological preservative during tea fermentation.

Although previous investigations identified *L. plantarum* strains from Ishizuchi-kurocha, namely IYO1511 and IYO1501, their potential to inhibit pathogen bacteria, produce lactic acid, encoded plantaricin gene have not yet been elucidated [5,20,21]. Therefore, this study aimed to examine the *L. plantarum* strains that were isolated from post-fermented tea of Ishizuchi-kurocha for their ability to inhibit pathogen bacteria, produce lactic acid, and detection of plantaricin gene. The sequencing of the gene clusters of each species was determined to identify their distinction. Previously research was found that the actual effects of the strains as food preservatives has also investigated for artificial fermentation of Ishizuchi-kurocha and it is successfully controlling the safety and quality of the product [5].

2. Materials and Methods

2.1 Isolation and Identification of *L. plantarum* strains

L. plantarum strain IYO1511 were formerly isolated from Ishizuchi-kurocha and the genome has been deposited at the GenBank with accession number BLLP00000000 [20]. Meanwhile, strain IYO1501 was newly isolated and identified by the extent of resemblance between 16S rRNA gene. The strains were grown in Man, Rogosa, and Sharpe (MRS) broth (Becton, Dickinson and Company, Franklin Lakes NJ, USA) at 37 °C for 24 hours in anaerobic condition.

2.2 Antibacterial effects of *L. plantarum* strains toward indicator bacteria

L. plantarum strains were cultivated in MRS Broth at 37 °C for 24 h in anaerobic condition with biomass as 10^8 CFU ml^{-1} and the antibacterial activities were measured using well diffusion method based on clear halo surrounding the wells. The solutions were centrifuged and filtered through 0.20 µm pore size filter (Advantec Toyo Roshi Kaisha Ltd., Tokyo, Japan). The indicator bacteria such as, *Escherichia coli* strain K12 JM109, *Bacillus subtilis*, and *Staphylococcus aureus* strain JM 20624 were cultured in Luria Bertani broth (Becton, Dickinson and Company, Franklin Lakes NJ, USA) at 30 °C for 12 h in aerobic condition. *L. plantarum* strain COY 2906 isolated from virgin coconut oil, was used as a positive control because the strain inhibited indicator bacteria, produced lactic acid and encoded *plnA, plnEF, plnN, plnJ* and *plnK* [9]. The wells on Mueller-Hinton agar (Becton, Dickinson and Company, Franklin Lakes NJ, USA) were cut and poured 100 µl of cell-free supernatant (CFS) of *L. plantarum* strains. The cultures were incubated at 30 °C for 24 h to determine the antimicrobial activity. The result that invented clear zone, indicating growth inhibition, and the determination were taken in triplicate for each experiment.

2.3 Lactic Acid Determination

The strains were cultivated in MRS Broth at 37 ˚C for 2, 4, 8, 16, 24, 48 and 72 hours in aerobic condition. A total of 5 microliters of CFS was added to 200 µl of a 0.2% solution of iron (III) chloride (Wako Pure Chemical Industries,

Osaka, Japan), and measured at wavelength 390 nm by spectrophotometer (Molecular Device SpectraMax M5, San Jose, US) followed spectrophotometric determination protocol [22]. The CFS of *L. plantarum* strains were filtered through 0.20 µm pore size filter. The solution was performed at 25 ± 5 °C and was stable for 30 minutes. Subsequently, lactic acid (Wako Pure Chemical Industries, Osaka, Japan) was used as a standard, with the equation of the calibration curve: y=0.5969x + 0.6972, with the correlation coefficient was 0.979. *L. plantarum* COY 2906 was used as a positive control [9], and the determinations were taken in triplicate for each experiment.

2.4 DNA Isolation from *L. plantarum* strains

L. plantarum strains were cultivated in MRS Broth at 37 ˚C for 18 hours in aerobic condition and the biomass of the cell was enumerated as 10^8 CFU ml^{-1}. The DNA of bacteria was extracted using the Extrap Soil DNA Kit Plus, version 2 (Nippon Steel Eco-Tech Corporation, Tokyo, Japan) according to the manufacturer's instructions. The quality of the DNA was running by 1% agarose gel in 1×TAE buffer. The gel was stained by Ethidium bromide and photographed under UV light.

2.5 Detection and Characterization Plantaricin of *L. plantarum* strains

Detection and characterization of plantaricin genes, *plnA, plnEF, plnN, plnJ,* and *plnK* of *L. plantarum* strains IYO1501 and IYO1511 were ensured by Real-Time qPCR (RT-PCR) (ABI Step One Plus, Thermo Fisher Scientific, Waltham, USA). The RT-PCR was carried out with a 10 µl final volume including 1 µl of DNA template, 1 µl of each primers (Table 1), 2 µl of nuclease free-water, and 5 µl of Power SYBR® Green PCR Master Mix (Applied Biosystems, Thermo Fisher Scientific, UK). The initial stage was carried out at 95 ºC for 10 minutes, conducted by 40 cycles at 95 ºC for 15 seconds, 60 ºC for 1 minute, and 72 ºC for 30 seconds. Subsequently, *16S rRNA* specific for lactic acid bacteria was elected as housekeeping genes based on the existing literature. These included *plnA, plnEF, plnN, plnJ,* and *plnK,* which were elected based on a screening test for plantaricin-encoding genes of the strains, where COY 2906 was used as a control [9]. The outcome was analyzed using the comparative critical threshold method (ΔCT), where the number of target genes was regulated to housekeeping gene and conducted twice for each gene.

2.6 DNA Sequencing

DNA sequences of *plnA, plnEF,* and *plnNJK* were read through DNA Sequencing Analytical service by the Division of Genomic Research, Gifu University using the Multi-capillary DNA Sequencer ABI Prism 3100/3130 Genetic Analyzer. Consensus and Open Reading Frame (ORF) were identified by the CLC Sequence Viewer 8.0. The computer alignment and BLAST (basic local alignment and search tool) analysis of the sequence were undertaken using BioEdit 7.2 for Windows 10.

2.7 Statistical Analysis

Measurement of antibacterial activities and lactic acid production were carried out in triplicate, however relative quantity of plantaricin were carried out in duplicate. All the results were expressed as the mean±standard deviation. The values in the tolerance test were analysed by one-way of variance followed Tukey's *post hoc* using RStudio version 4.1.3 suitable for windows 10. Statistical significance was set at $P < 0.05$.

3. Results and Discussion

3.1 Antibacterial activities of *L. plantarum* strains

Antibacterial activity was reported as an effective way to eliminate or inhibit pathogens, and it was evaluated by the existence of a clear halo surrounding bacterial cell extract from cells collected at 24h, and the clear zones were considered positive. Strain COY 2906 was reported inhibit these indicator bacteria and considered positive control [9]. Figure 1 showed that the CFS of *L. plantarum* strain IYO1501 and strain IYO1511 were positively inhibited the pathogenic bacteria. *L. plantarum* strain IYO1501 showed better inhibition on *S. aureus* JCM 20624 than strain IYO1511. However, strain IYO1511 showed better inhibition on *E. coli* strain K12 JM109 and *B. subtilis*.

Table 1. Real-Time PCR primer sequence and amplicon size detection and characterization of plantaricin genes

Target Genes	Sequence	Amplicon size	Reference
Housekeeping			
16S rRNA_F	GATGCATAGCCGACCTGAGA	114	[23]
16S rRNA_R	CTCCGTCAGACTTTCGTCCA		
Plantaricins			
plnA_F	AAAATTCAAATTAAAGGTATGAAGCAA	108	[24]
plnA_R	CCCCATCTGCAAAGAATACG		
plnEF_F	GTTTTAATCGGGGCGGTTAT	85	
plnEF_R	ATACCACGAATGCCTGCAAC		
plnN_F	GCCGGGTTAGGTATCGAAAT	102	
plnN_R	TCCCAGCAATGTAAGGCTCT		
plnJ_F	TAAGTTGAACGGGGTTGTTG	102	
plnJ_R	TAACGACGGATTGCTCTGC		
plnK_F	TTCTGGTAACCGTCGGAGTC	97	
plnK_R	ATCCCTTGAACCACCAAGC		

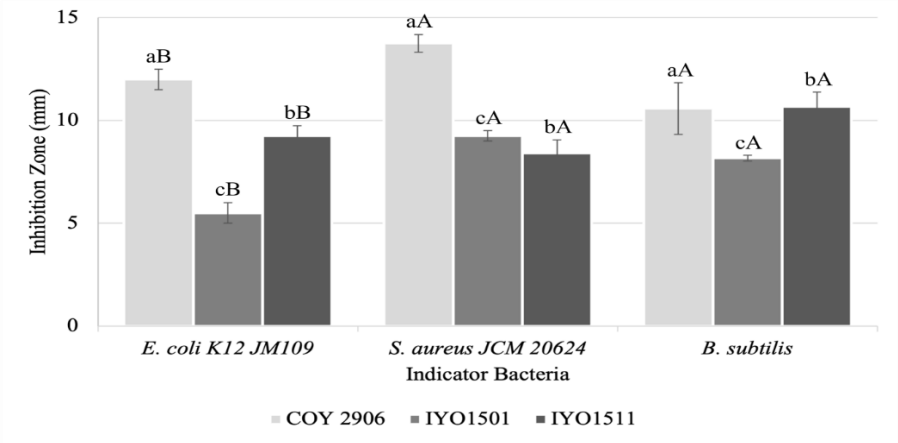

Figure 1. The inhibition zone of *L. plantarum* strains againts the indicator bacteria; *E. coli* K12 JM109, *S. aureus* JCM 20624, *B. subtilis.* The data were expressed as mean±standard error with p<0.05 (n=3). [a-c]Significantly different between antibacterial activity and *L. plantarum* strains by Tukey's test; [A-C]Significantly different between antibacterial activity and indicator bacteria by Tukey's test.

3.2 Lactic Acid Production by *L. plantarum* strains

Lactic acid is a primary antimicrobial agent in food fermentation produced by LAB, which is used as bactericidal and bacteriostatic in agriculture as well as food production [12]. The best time to harvest the lactic acid from *L. plantarum* strains was within 16-72 hours. The lactic acid produced by *L. plantarum* strains isolated from Ishizuchi-kurocha can prevent food spoilage and give an acidic taste to post-fermented tea [24] as shown in Figure 2. The strain IYO1511 produced the highest value with incubation time 72 and 24 hours, namely 3.4135±0.180% and 3.401±0.140%, respectively.

3.3 Detection and Characterization of Plantaricin Genes of *L.* plantarum strains

The plantaricin genes were detected and characterized on *L. plantarum* strains and expected as antimicrobial peptides. Moreover, the genes devised were *plnA, plnEF, plnN, plnJ* and *plnK.* The relative quantity of plantaricin was harvested at the beginning of the stationary phase to determine the genes involved in bioactivity of plantaricin. Figure 3 showed the plantaricin genes of *plnA, plnEF, plnN, plnJ* and *plnK*, which were present in *L. plantarum* strain IYO1511. However, *L. plantarum* strain IYO1501 did not have *plnA* and *plnK* genes. The *plnEF, plnN,* and *plnJ* of *L. plantarum* strain IYO1511 indicated higher relative quantity plantaricin genes than IYO1501 on their DNA.

Previous studies reported that *plnA*, *plnEF*, and *plnNJK* have antimicrobial activity against food spoilage organisms [25-27]. The two peptides showed a feeble antimicrobial activity; however, their combination can increase antimicrobial potential in 1000 folds [26,28]. These results were suspected to be a combined peptide that exhibited higher antimicrobial activity toward food spoilage organisms without changing the taste and characteristics of food. Further discuss is needed to clarify antimicrobial active peptides of plantaricin and the mode of action of each gene to induce significant membrane disruption that causes cell death.

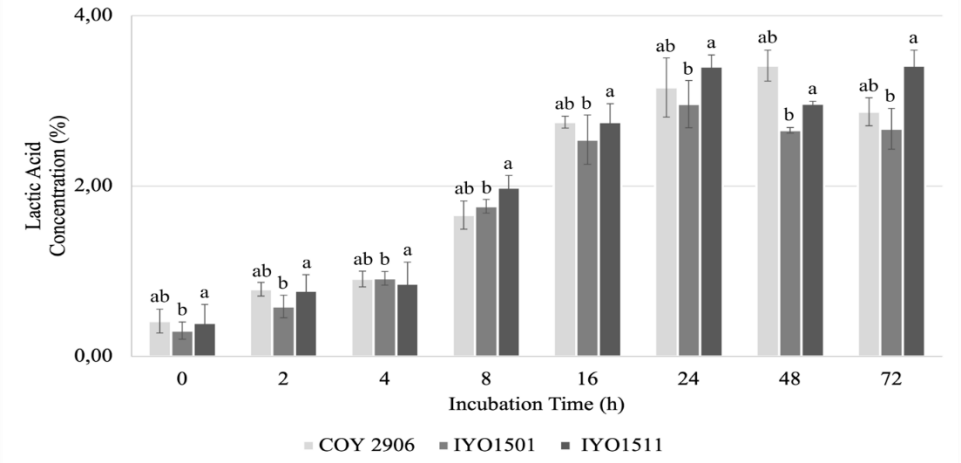

Figure 2. Lactic acid production by *L. plantarum* strains. The data were expressed as mean±standard error with p<0.05 (n=3). [a-c]Significantly different between lactic acid concentration and *L. plantarum* strains by Tukey's test. The time incubation and lactic acid concentration are significantly different, however 16h, 24h, 48h and 72h are not significantly different.

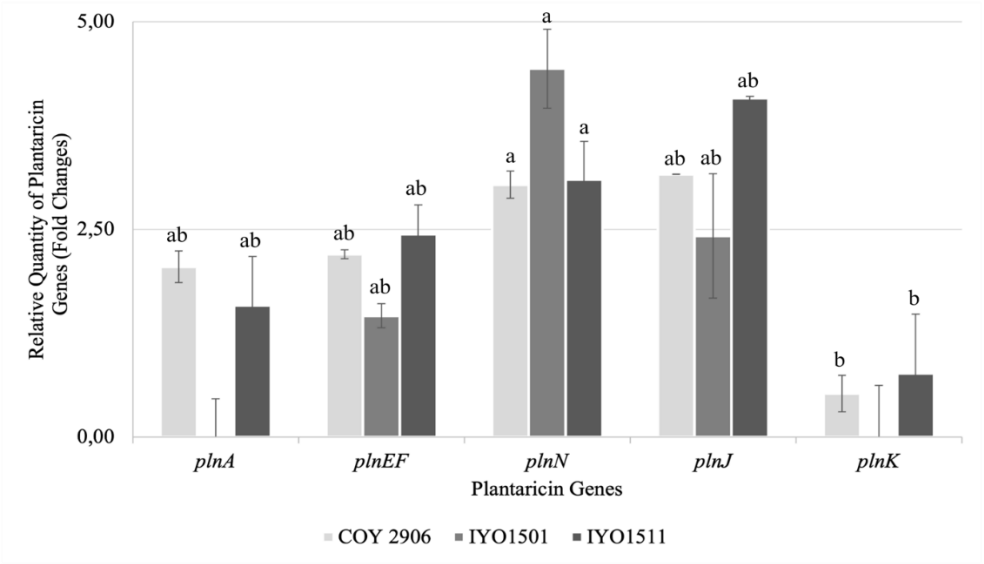

Figure 3. The gene amount of plantaricin genes on *L. plantarum* strains. The data were expressed as mean±standard error with $p<0.05$ (n=2). [a-c]Significantly different between relative quantity of plantaricin genes and *L. plantarum* strains by Tukey's test. However, the relative quantity of plantaricin genes and *L. plantarum* strains are not significantly different.

a.

b.

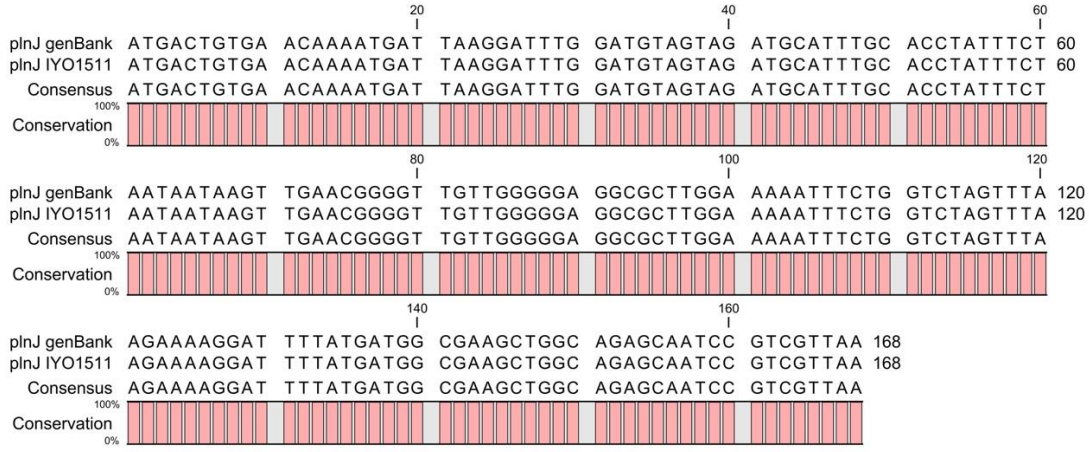

Figure 4. The *similarity homology between plnA* and *plnJ* from GenBank and *L. plantarum* strain IYO1511.

3.3 The Heterologous of Plantaricin Genes from *L. plantarum* strain IYO1501 and IYO1511

The genes of plantaricin from *L. plantarum* strain IYO1501 and IYO1511 were sequenced to investigate the heterologous gene clusters of each species. The PCR product of *plnA*, *plnEF*, and *plnNJK* was also amplified and sequenced from the DNA of *L. plantarum* strains. Furthermore, the contig of *plnA* and *plnEF* sequence on *L. plantarum* strain was read as ~550bp, while *plnNJK* of *L. plantarum* IYO1511 sequence was ~1500bp. The sequences were analysed using CLC Sequence Viewer 8.0 and Bioedit 7.2 for windows and Bioedit ClustalW Multiple Alignment was used for sequence alignment. Plantaricin from GenBank was compared with the plantaricin from *L. plantarum* strains IYO1501 and IYO1511. The sequences analysis in Figure 4 and 5 represented no differences between *plnA*, *plnEF*, and *plnJ* GenBank. Based on observations, it was discovered that *L. plantarum* IYO1511 and all sequences were identical with 100% between GenBank and *L. plantarum* IYO1511. The obtained *plnN* sequences were compared to GenBank and *L. plantarum* strains IYO1511.

The obtained *plnEF* sequences were compared using GenBank and *L. plantarum* strains IYO1501. As shown in Figure 5, the sequences presented extra base pairs inserted into the DNA of *L. plantarum* IYO1501. The sequences were shown in Figure 6, which presented extra base pairs inserted into the DNA of *L. plantarum* IYO1511. This led to a frameshift in which subsequent codons reads were altered and the entire amino acid sequence altered. The protein purification and amino acid sequencing from *plnEF* and *plnN* need to be carried out and compared with the database from GenBank. However, the *plnK* of *L. plantarum* IYO1511 was not analysed due to a truncated sequence. The mechanisms employed in the inhibition and diminishing of pathogenic bacteria depended on the characteristics of the plantaricin. For example, various classes of plantaricin have different mechanism [12]. The differences in location can provide variation in gene structure, protein immunities, and secretion genes. Similarly, different strains also produced diverse plantaricin structural genes.

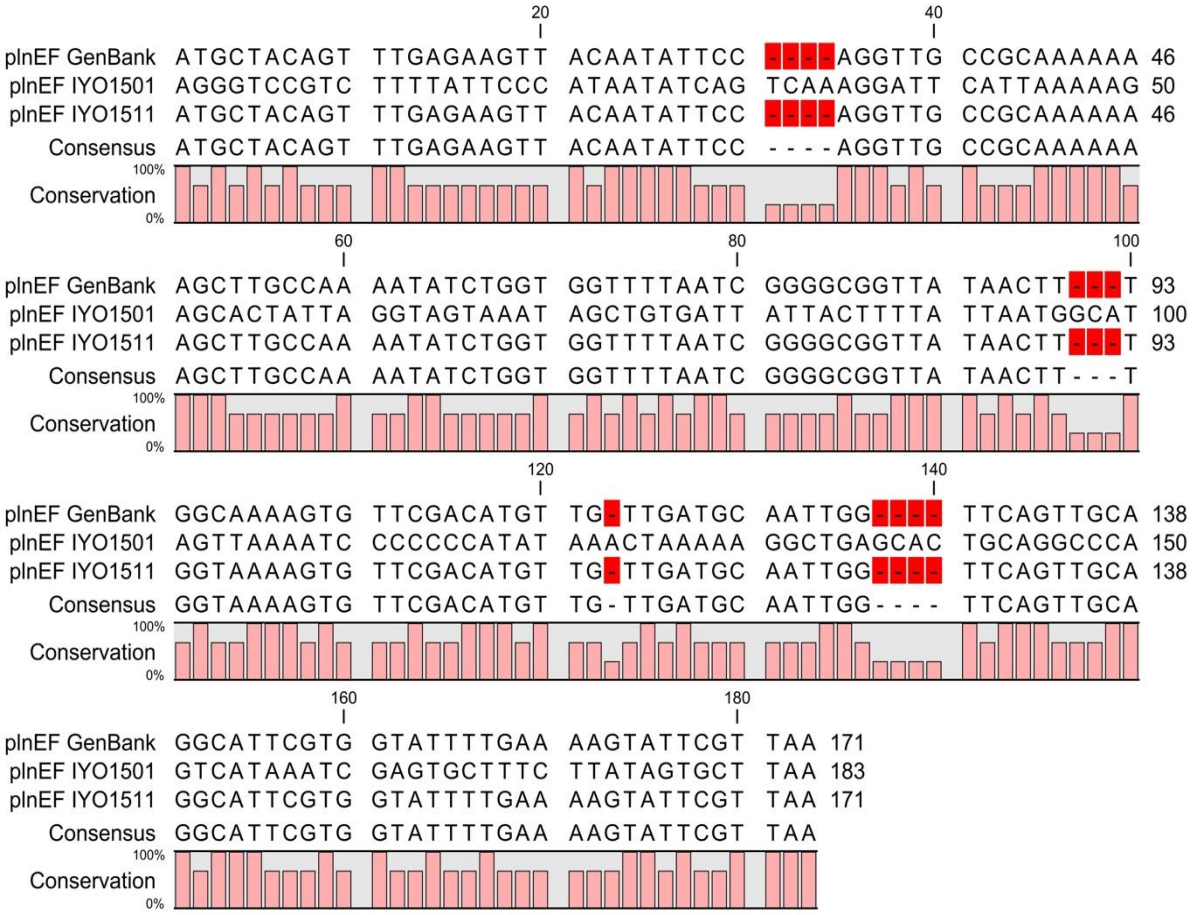

Figure 5. The similarity homology between *plnEF* from GenBank and *plnEF* from *L. plantarum* strains. The red symbol explained missing base pairs sequenced.

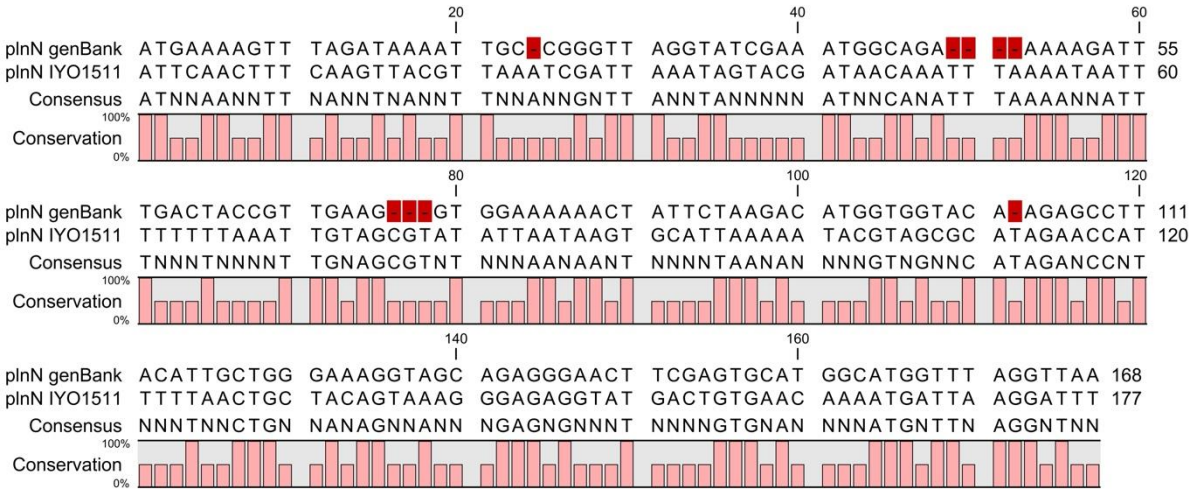

Figure 6. The *similarity homology between plnN* from GenBank and *plnN* from *L. plantarum* strains. The red symbol explained missing base pairs sequenced.

4. Conclusion

Ishizuchi-kurocha is produced by unique manufacturing processes and fermented fermented with a spontaneous fermentation. There are two kinds of microorganism embroiled in fermentation process. These include the primary fermentation, which is the fermenting of tea leaves by fungi and followed by the secondary step with the anaerobic condition by lactic acid bacteria [21]. *L. plantarum* is a predominant lactic acid bacterium in Ishizuchi-kurocha [7] and had been identified from anaerobically fermented tea of Ishizuchi-kurocha tea leaves samples, namely IYO1511 and IYO1501. The capacities of strains to has antibacterial activities, produce lactic acid and plantaricin genes have not yet been elucidated. The investigations showed that *L. plantarum* produced organic and fatty acids, ammonia, hydrogen peroxide, diacetyl and bacteriocin as bactericidal and bacteriostatic to survive [9]. *L. plantarum* strains successfully inhibited the growth of *E. coli* strain K12 JM109, *B. subtilis* and *S. aureus* strain JM 206244. The results showed that strain IYO1511 better inhibit Gram positive and negative bacteria, and strain IYO1501 better inhibit Gram-positive bacteria. *L. plantarum* isolated from sourdough was reported as bacteriostatic of *E. coli*, *S. aureus* and, *L. monocytogenes* [29]. Meanwhile, *L. plantarum* strain IYO1511 produced higher lactic acid that IYO1501. *L. plantarum* strain DY-6 was reported produce higher lactic acid, than other acid, such as acetic acid, propionic acid, decanoic acid, and octanoic acid which showed antibacterial activity against *Escherichia coli* [30].

Approximately 5% of lactic acid was reported to significantly reduce *Salmonella* spp. in chicken thighs and breasts, without any effect on physical properties such as the color of skin. It also reduced intracellular pH and disrupted the cytoplasmic membrane of *Salmonella* spp. [31]. Lactic acid can also alter cell membrane permeability and generate hydroxyl radicals based on intracellular irons ions that cause DNA demage and cell death [16]. *L. plantarum* strains isolated from Ishizuchi-kurocha can prevent food spoilage, change the component composition as well as taste enhancement of Ishizuchi-kurocha, and improve the shelf-life of tea by increase acid [21]. Therefore, maintaining the efficient driving of the fermentation process is crucial in Ishizuchi-kurocha.

The plantaricin gene at *L. plantarum* strains was suspected to be expressed through the process of bacteriocins synthesis [24], which has antimicrobial peptide activity as a bio-preservative on post-fermented tea. It was discovered that plantaricin has high specificity for some clinically pathogenic bacteria and provides antimicrobial activity at relatively high temperatures, acidic, or alkaline conditions, and at greater levels of salinity which provide benefits to the food industry [12]. In this study, the analysis of the genes showed that *L. plantarum* strain IYO1511 has plantaricin genes, *plnA*, *plnEF*, *plnN*, *plnJ* and *plnK*, while IYO1501 did not have *plnA* and *plnK* genes. *L. plantarum* RUB1 isolated from traditional fermented koumiss was reported to encode five operons included *plnA*, *plnN*, *plnEF*, and *plnJK* related to bacteriocin synthesis against *Listeria monocytogenes* ATCC 19111, *Pseudomonas fluorescens* ATCC 13525, *Salmonella* serotype Typhimurium ATCC 14028, *Streptococcus thermophilus* ATCC 14485, and *L. bulgaricus* ATCC 11842 [32].

The mechanisms of action employed in the inhibition and diminishing of pathogenic bacteria depend on the particularity of the plantaricin. For example, various classes of plantaricin have a variety of actions. Insertion of *plnA* inside of the cytoplasmic membrane of the target cells promotes membrane depolarization and the cell death [33]. The *plnEF* can permeabilize the cell membrane, which causes small molecules transfer out and in, leading to the dismiss of the cell proton motive force (PMF), including transmembrane electrical potential ($\Delta\Psi$) and pH gradient (ΔpH) [27]. The mechanism of *plnNJK* action of *L.*

plantarum strain C11 cells interrupted the integrity of the cell membrane and the formation of holes in the target cells [34]. Complementary peptides were leastwise 10^3 times more effective when they were combined compared to their presence [27,28]. These mechanisms in action can provide bio-preservatives for Ishizuchi-kurocha and are called silent killers without changing the characteristics of Ishizuchi-kurocha. Nisin and lactic acid were reported to synergize with mild heat and hydrostatic pressure can reduce *Bacillus amyloliquefaciens*, *Geobacillus stearothermophilus*, and *Bacillus atrophaeus* endospores to >4 log CFU ml^{-1} [35]. L-lactic acid and plantaricin EF released LPS and bond the cell membrane, inhibiting protein synthesis, protein folding and DNA replication of *A. hydrophila*. The side effect was a significant change in morphological and intercellular, including inner membrane interference, Structural DNA modification, abnormal cell elongation and coagulation of the cytoplasm of *A. hydrophila* [15]. Although the lactic acid and plantaricin of *L. plantarum* are expected to have a synergy in inhibiting food spoilage bacteria, further study on the mode in action is recommended.

The gene of plantaricin from *L. plantarum* strains IYO1501 and IYO1511 were sequenced to know the heterologous gene clusters of each species. The differences in location may provide variations in gene structure, protein immunities, and secretion genes [36]. Furthermore, different strains produced various plantaricin structural genes. The *plnEF* sequence of *L. plantarum* strains IYO1501 presents extra-base pairs inserted into the DNA of strains. The *plnN* sequence of *L. plantarum* strains IYO1511 also showed the extra base pairs inserted into the DNA. This amino acid needs more analysis on its sequence and purification to see the differences. The use of the current sequencing showed that there is a significant homolog/similarity with class II bacteriocins. *plnEF* was reported to be sequenced to investigate a potential coded protein and found that there were two insertions of T and C at positions 675 and 703 on *L. plantarum* strain BL1 [37].

The unique strains of *L. plantarum* contribute to the success of the fermentation process and the unique taste of the post-fermented teas. During this process, the addition of *L. plantarum* strain IYO1511 controlled the microbial composition [5]. Lactic acid concentration and plantaricin production are some of the important factors considered in the fermentation process. These strains can also be necessary to suppose the biological characteristics, including their antioxidant activity. For future study, it is recommended to elucidate the diversity of *L. plantarum* and control the fermentation of Ishizuchi-kurocha.

5. Acknowledgements

This study showed that novel *L. plantarum* strains isolated from Ishizuchi-kurocha have antibacterial activities and produced lactic acid and plantaricin, where IYO1511 gave a higher value than IYO1501. *L. plantarum* strains can be used as food preservative for artificial fermentation to control the safety and quality of the product of Ishizuchi-kurocha.

6. Conflict of Interest

The authors report no conflicts of interest.

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8. Conflict of Interest

This manuscript has not been published and is not under consideration for publication elsewhere. The authors have declared no conflicts of interest.

9. Authors Contributions

Conceptualization, Y.S., N.R., T.S.E., and I.H; methodology, Y.S., T.H., and J.L.; software, Y.S. and T.H.; validation, I.H.; writing-original draft preparation, Y.S., J.L., T.H., S.F., and I.H.; writing—review and editing, Y.S., J.L., T.H., S.F., and I.H.; supervision, Y.S. and I.H.; project administration, Y.S.; funding acquisition, Y.S.

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مشخصات ژن‌های پلانتاریسین و تولید لاکتیک اسید توسط سویه‌های *لاکتی‌پلانتی‌باسیلوس پلانتاروم* جدا شده از Ishizuchi-Kurocha

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چکیده

سابقه و هدف: Ishizuchi-kurocha چای پس از تخمیر دو نوع میکروارگانیسم اصلی یعنی قارچ و باکتری لاکتیک اسید، به ترتیب تخمیر اولیه و ثانویه می‌باشد. بنابراین، این تحقیق با هدف تایید نقش *لاکتی‌پلانتی‌باسیلوس پلانتاروم* در تخمیر ثانویه Ishizuchi-kurocha و اثر ضد باکتریایی ناشی از تولید لاکتیک اسید و شناسایی ژن پلانتاریسین انجام شد.

مواد و روش ها: میزان تاثیر ضدمیکروبی با استفاده از روش انتشار چاهی تخمین زده شد. لاکتیک اسید با روش طیف سنجی اندازه‌گیری شد. توالی ژن‌های پلانتاریسین توسط آنالیز واکنش زنجیره‌ای پلیمراز همزمان (Real-Time qPCR)[1] تایید شد. ژن ها از طریق سرویس تحلیل توالی DNA توسط بخش تحقیقات ژنومی، دانشگاه گیفو با استفاده از آنالایزر ژنتیکی ABI Prism 3100/3130 DNA Sequencer چند ستون مویین تعیین توالی شدند و داده ها توسط CLC Sequence Viewer 8.0 و BioEdit 7.2 تجزیه و تحلیل شدند. تجزیه و تحلیل آماری با استفاده از واریانس یک طرفه و به دنبال آزمون تعقیبی توکی با استفاده از RStudio نسخه ۴،۱،۳ مورد ارزیابی قرار گرفت.

یافته‌ها و نتیجه‌گیری: سویه *لاکتی‌پلانتی‌با سیلوس پلانتاروم* IYO1511 فعالیت ضد باکتریایی بیشتری نسبت به سویه IYO1501 دارد. علاوه بر این، سویه *لاکتی‌پلانتی‌با سیلوس پلانتاروم* IYO1511 لاکتیک اسید بالاتری نسبت به سویه IYO1501 تولید کرد و دارای ژن‌های پلانتاریسین، plnA، plnEF، plnN، plnJ و plnK بود. درحالی‌که، سویه *لاکتی‌پلانتی‌با سیلوس پلانتاروم* IYO1501 فقط کدهای ژنتیکی plnEF، plnN و plnJ را داشت. ژن‌های پلانتاریسین از سویه های IYO1501 و IYO1511 برای شناسایی خوشه‌های ژن هترولوگ هر گونه توالی‌یابی شدند. مشخص شد که ژن‌های plnJ ، plnA، plnEF، *لاکتی‌پلانتی‌باسیلوس پلانتاروم* IYO1511 ، شباهت ۱۰۰ درصدی را با بانک ژن نشان دادند. plnN سویه *لاکتی‌پلانتی‌با سیلوس پلانتاروم* IYO1511 و plnEF سویه IYO1501 جفت‌های باز اضافی را در DNA وارد می‌کنند. از سویه‌های *لاکتی‌پلانتی‌باسیلوس پلانتاروم* می‌توان به‌عنوان نگهدارنده مواد غذایی برای تخمیر مصنوعی برای کنترل ایمنی و کیفیت محصول Ishizuchi-kurocha استفاده کرد. انتظار می‌رود که لاکتیک اسید و پلانتاریسین با مهار بیماری‌زایی و فساد باکتری‌ها، طعم اسیدی منحصر به فردی به‌عنوان رایحه ایشیزوچی-کوروچا تولید کنند.

تعارض منافع: نویسندگان اعلام می‌کنند که هیچ نوع تعارض منافعی مرتبط با انتشار این مقاله ندارند.

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- Ishizuchi-kurocha
- لاکتیک اسید
- *لاکتی‌پلانتی‌باسیلوس پلانتاروم*
- ژن‌های پلانتاریسین

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