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Molecular Detection of the Plantaricin A (plnA) Gene in the *Lactobacillus casei* Group

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Abstract: The plantaricin A (plnA) gene encodes a pheromone peptide that induces the synthesis of bacteriocins in *Lactobacillus plantarum* but can also be found in other species. *Lactobacillus casei*, *Lactobacillus paracasei*, and *Lactobacillus rhamnosus* are the most commonly studied and used probiotics for the synthesis of antimicrobial compounds such as bacteriocins. This study specifically focused on detecting the presence of the plnA gene, a single gene associated with plantaricin synthesis, in the *L. casei* group. Detection of the plnA gene was carried out through bacterial culturing, direct colony PCR, PCR amplification using plnA-F and plnA-R specific primers, electrophoresis, sequencing, and sequence analysis using BLAST. The presence of bands in the electrophoresis of PCR results revealed that the plnA gene was not detected in *L. rhamnosus*, but was found in *L. casei* and L. *paracasei*. Sequencing analysis of the plnA gene from *L. casei* and *L. paracasei* revealed 99.56% and 100% similarity with the plnA gene from *L. plantarum* EG.LP.18, respectively. The plnA gene found in *Lactobacillus casei* and *Lactobacillus paracasei* can be used to produce bacteriocins, which are antibacterial compounds.

Keywords: anti-bacterial agents; bacteriocins; *Lactobacillus casei*; plnA gene.

1. Introduction

Food spoilage caused by microbial activity continues to be a major problem in the food industry [1]. Microbial spoilage reduces shelf life, texture, and product quality, causing significant economic losses [2]. According to Dong *et al*., microbial spoilage wastes up to 1.3 billion tons of food products each year [3]. Chemicals are often used in the food industry to inhibit the growth of spoilage microbes and extend the shelf life of food [4]. However, chemicals used as food preservatives can be toxic and have harmful effects on the body.

Many natural preservatives derived from microorganisms have been developed in recent years, one of which is the use of bacteriocins [5]. Bacteriocins are peptides or proteins synthesized by ribosomes and released extracellularly [6], and they exhibit bactericidal activity [7]. Bacteriocins have been used in many countries to inhibit the growth of food spoilage microorganisms, making them useful as natural preservatives in food [8]. Bacteriocins can also

be used to suppress the growth of food spoilage microorganisms and pathogens, thereby prolonging the shelf life of food products [9].

According to Mokoena, bacteriocins can be produced by several species of lactic acid bacteria (LAB) [10]. Bacteriocins derived from Lactobacillus strains are commonly used in food preservation. Lactobacillus is a probiotic LAB group that produces a variety of bacteriocins used in food preservation and has the potential to promote health [11]. *Lactobacillus casei*, *Lactobacillus paracasei*, and *Lactobacillus rhamnosus* are the most well-studied and widely used members of the *Lactobacillus casei* group as probiotics, according to Hill *et al*. [12]. *Lactobacillus casei*, *Lactobacillus paracasei*, and *Lactobacillus rhamnosus* are phylogenetically and phenotypically related; thus, they are grouped as the *L. casei* group [13].

Plantaricin is a gene that encodes a pheromone peptide that induces bacteriocin synthesis in the *Lactobacillus* genus, particularly in many *Lactobacillus plantarum* species. Plantaricin has the potential to be used as a food biopreservative due to its ability to inhibit or kill pathogenic bacteria, which has garnered significant interest [14]. According to Echegaray *et al*., applying plantaricins to fresh fish can extend food shelf life without affecting its nutritional content [15]. Based on genetic analysis, bacteriocins from *L. plantarum* are classified as plantaricins, which are encoded by several plantaricin (pln) genes, including the plantaricin A (plnA) gene [6].

PlnA gene analysis has only been conducted on *L. plantarum* species, with limited information available on the *L. casei* group. Preliminary experiments revealed that the *L. casei* group exhibits antibacterial activity against *E. coli* and *S. aureus*. According to Oberg *et al*., the *L. casei* group is genetically similar to *L. plantarum* [16]. This suggests that the plnA gene may also be present in the *L. casei* group. The objective of this study was to detect the plantaricin A gene encoding bacteriocin in the *L. casei* group.

2. Materials and Methods

2.1. Materials.

The materials used in this study were isolates of *L. casei* group (*L. casei*, *L. paracasei*, and *L. rhamnosus*) [17], MRSA (*de Man Rogosa Sharpe Agar*) (Merck) and MRSB (*de Mann Rogosa Sharpe Broth*) media (Merck), 70% alcohol (OneMed), PCR Master Mix (Invitrogen), DNA ladder (Invitrogen), agarose, TBE buffer, Ethidium Bromide (EtBr), loading dye, plnA-specific primers F (5'-GTACAGTACTAATGGGAG-3') and R (5'-CTTACGCCAATCTATACG-3') [18].

2.2. Methods.

2.2.1. Rejuvenation and culture preparation of *L. casei* group.

Rejuvenation of *L. casei* group isolates was carried out on MRSA media. Sterilized MRSA medium was placed into a petri dish and allowed to settle until solid. Isolates from the *L. casei* group (*L. casei*, *L. paracasei*, and *L. rhamnosus*) were cultured on MRSA media using the scratch pad method. Furthermore, the incubation period was 48 hours at 37°C [9].

2.2.2. Direct PCR amplification.

The plantaricin A (plnA) gene was amplified in the *L. casei* group using the direct PCR Amplification method according to Ben-Amar *et al*. [19]. A single bacterial colony was placed

at the bottom of the microtube and then heated in a 60°C water bath for 10 minutes before being placed in ice for 2 minutes. Bacterial colonies were given 10 µL (2x) PCR Master mix, 1 µL reverse primer, 1 µL forward primer, and free water to a final volume of 20 µL.

The amplification process used primers (plnA) F (5'-GTACAGTACTAATGGGAG-3') and R (5'-CTTACGCCAATCTATACG-3'). The initiation stage was carried out at 98°C for 5 minutes, denaturation at 94°C for 1 minute, annealing at 53°C for 1 minute, extension at 72°C for 1 minute, and final extension at 72°C for 1 minute. The denaturation, annealing, extension, and final extension stages were carried out with 35 cycles [20].

2.2.3. Electrophoresis of PCR products.

Electrophoresis of plnA gene PCR amplification products was performed using 1,5% agarose gel. Agarose gel was made by dissolving 0,3 g agarose powder and 20 mL of 1X TBE buffer. Agarose powder was microwaved until dissolved and clear. The agarose gel solution was poured into gel molds and left to harden at room temperature. Agarose gel was then transferred into the electrophoresis chamber, and TBE 1X buffer was poured until it was completely submerged. Each agarose gel well was filled with 6 µL of sample consisting of 3 µL PCR product and 3 µL loading dye (1:1). Electrophoresis was performed for 40 minutes with a voltage of 100 volts. Agarose gel was stained in EtBr solution for 15 minutes. The electrophoresis results were then observed under the gel doc UV transilluminator [21].

2.2.4. Sequencing of Plantaricin A gene.

Electrophoresis of PCR products that showed positive results was sequenced to determine the nucleotide base sequence of the plantaricin A (plnA) gene. PCR samples were then sent to Bioneer, Korea. PCR samples were purified to obtain the DNA template. The sequencing data were then used to analyze the level of similarity with the gene sequence already available in the NCBI GenBank database.

Data analysis was carried out descriptively and qualitatively based on the presence of plantaricin A gene DNA bands. The size of the DNA fragment of the L. casei group with the selected PCR amplification results was ±450 bp. Sequencing data were analyzed for similarity using NCBI with the BLAST program (https://blast.ncbi.nlm.nih.gov).

3. Results and Discussion

3.1. PCR amplification of plantaricin A gene.

Detection of the plantaricin A (plnA) gene encoding bacteriocin in L. casei was performed through direct colony PCR amplification. The number of bacterial cells added directly to the PCR reaction is an important factor in direct colony PCR. Bacterial cells should be added in the least number possible in order to minimize inhibitors that could cause the PCR amplification process to fail. The direct colony PCR technique, according to Ben-Amar *et al*., is one of the most useful molecular techniques used in various areas of biological research and diagnostics [19]. When compared to conventional PCR, this approach offers significant advantages in terms of speed and efficiency. Most traditional PCR techniques necessitate base lysis with hazardous chemical solvents and take a long time to extract DNA. Direct colony PCR is widely used in a variety of research fields, including food control and environmental

microbiology, human health and genetic disease diagnosis, agriculture, and plant biotechnology.

The PCR amplification process was performed using specific primers plnA F and R to amplify the targeted gene fragments. Electrophoresis was used to visualize the PCR amplification results on an agarose gel. Positive PCR amplification of the plnA gene from the L. casei group was shown by the appearance of bands on the agarose electrophoresis gel image (Figure 1). Two species from the *L. casei* group showed results that were positive for PCR amplification of the plnA gene in this study.

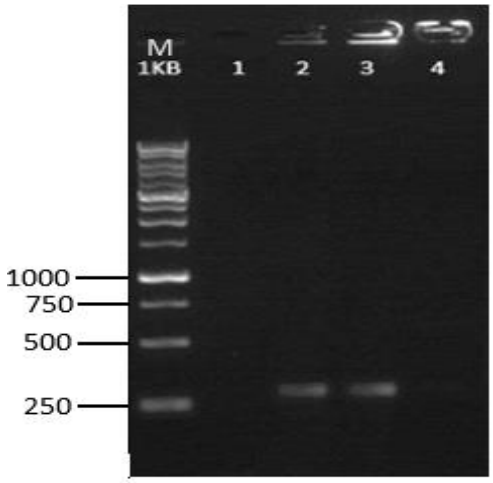

Figure 1. Visualization of plnA gene PCR amplification results on agarose gel. M: Marker DNA; 1: Negative control; *2: L. casei*; *3: L. paracasei*; *4: L. rhamnosus*.

In *L. casei* and *L. paracasei*, PCR amplification using the specific primers plnA-F and plnA-R yielded DNA fragments of approximately 300 bp. This differs from the expected DNA fragment size of ~450 bp observed in *L. plantarum*, where the same primer pair is known to span the full coding region of the plnA gene. The smaller fragment size detected in *L. casei* and *L. paracasei* may be attributed to differences in the genomic context of the plnA gene, partial sequence conservation, or potential truncation. Another possibility is that mutations at primer binding sites may have affected amplification efficiency, leading to a shorter product. This size variation is consistent with previous reports: El Issaoui *et al*. [20] observed a ~455 bp DNA fragment of plnA in *L. plantarum* 11, while Mustopa *et al*. [22] reported a ~300 bp fragment in *L. plantarum* S34. These findings suggest that DNA fragment length may vary depending on strain-specific genomic differences and the degree of sequence conservation in the target region.

The detection of the plnA gene in the *L. casei* group demonstrates that *L. casei* and *L. paracasei* both possess a bacteriocin-coding gene. The presence of the gene revealed that *L. casei* and *L. paracasei* exhibit antibacterial activity since both produce antimicrobial substances in the form of bacteriocins. The plnA gene is a pheromone peptide-coding gene that works as an inducing factor in bacteriocin formation and is used to identify bacteriocins in the *L. casei* group. Plantaricin A is a well-known pheromone peptide that stimulates plantaricin bacteriocin synthesis in L. plantarum [15].

Lactobacillus casei and *L. plantarum* as probiotic bacteria are often found in the same living environment. They are often found together as members of the human gastrointestinal microbiota. In the gastrointestinal tract, there are various ecological niches and various Lactobacillus, including *L. casei* and *L. plantarum* [22]. According to Wang *et al*., *L. casei*

species have good adaptability in various environments [23]. There are eight genes found in *L. casei* as well as other gut microbiota, including *L. plantarum* and several other Lactobacillus species, in the gastrointestinal tract. The eight genes are plnA, plnB, plnC, plnD, plnI, plnF, plnE, and plnG, which encode bacteriocins and are regulated by operons. The most widely studied bacteriocin in *L. casei* is casein [8]. This does not limit the presence of other bacteriocin-encoding genes. Therefore, it is estimated that *L. casei* has a wide genetic diversity. The similarity of the plnA gene as a bacteriocin encoder in *L. plantarum* was also found in *L. casei* and *L. paracasei* in this study, so it is thought to be in the same ecological niche. Bacteriocin genetic elements are most often plasmids, but can also be chromosomes. This explains that the same bacteriocin can be produced by different species [24].

Caseicin is the name of the bacteriocin present in *L. casei*. According to Noroozi *et al*., *L. casei* has class III bacteriocins [25]. This bacteriocin is heat-labile, has a high molecular weight (>30 kDa), and is unmodified. Caseicin TN-2's antibacterial activity in *L. casei* was maintained over a wide pH range and a 20-minute heat treatment at 121°C. Furthermore, it is sensitive to proteases, such as trypsin and papain. Caseicin has a broad antibacterial range that includes some antibiotic-resistant strains of Gram-positive and Gram-negative foodborne pathogens [26]. The ability of the *L. casei* group to produce caseicin requires additional research.

Previous research found *L. rhamnosus* bacteria to exhibit antibiotic activity against *E. coli* and *S. aureus*; however, PCR amplification of the plnA gene yielded negative results (Figure 1). These findings could be explained by the presence of other bacteriocin genes in *L. rhamnosus* that were not found with the plnA gene in this study. Similar findings were made in the study by Bu *et al*. [27], which revealed that just 6% of all Lactobacillus isolates possessed bacteriocin coding genes, despite the fact that 40% of all isolates tested positive for phenotypic testing. This distinction is possible because several bacteriocin-coding genes differ between Lactobacillus species.

The presence of the plnA gene in the *L. casei* group indicates that the production of certain bacteriocins is not necessarily associated with a single species. The plnA gene that has been described as encoding bacteriocin in *L. plantarum* was also found in *L. casei* and *L. paracasei* in this study. Therefore, other bacteriocin-encoding genes are expected to be found in *L. rhamnosus*. A number of other bacteriocins that have been found, such as curvasin from *L. curvatus*, acidosin from *L. acidophilus*, brevicin from *L. brevis*, and sakacin from *L. sakei* [28], can be used in *L. rhamnosus*. According to Perez *et al*., the production of certain bacteriocins does not have to be associated with one species nor limited to organisms that occupy the same environment [29]. The curvasin A gene encoding bacteriocin found in *L. curvatus* LTH1174 is also produced by *L. sake* CTC494. According to Zhao *et al*., lactosin 160 isolated from *L. rhamnosus* zrx01 has antibacterial effects on *Micrococcus luteus* and other pathogenic bacteria [30].

Furthermore, the antibacterial activity of *L. casei*, *L. paracasei*, and *L. rhamnosus* could be attributed to the formation of organic acids. The presence of organic acid production alone or in combination with bacteriocin formation can result in the bactericidal impact of an antimicrobial activity. According to the findings of this study, the antibacterial activity in *L. casei* and *L. paracasei* was derived from the formation of bacteriocins and organic acids. Lactic acid bacteria (LAB) from the *L. casei* group, according to Walter *et al*., can create antimicrobial substances, such as organic acids (lactic, citric, acetic, fumaric, and malic), hydrogen peroxide, diacetyl, ethanol, and bacteriocins [31].

Antimicrobial substances are classified as either ribosomally or non-ribosomally generated peptides [32]. Bacteriocins are peptides that are ribosomally produced and can be released into the extracellular environment. However, not all LAB species can produce antibacterial bacteriocins [33]. Meanwhile, LAB fermentation produces various antimicrobial substances such as organic acids [34].

3.2. Sequencing of plantaricin A gene.

The PCR amplification results of the *plnA* gene from *L. casei* and *L. paracasei* showed positive bands, which were subsequently subjected to sequencing analysis. Based on the sequencing results, partial coding sequences (CDS) of the *plnA* gene from *L. casei* and *L. paracasei* were obtained (Figure 2). These sequences were analyzed using the Basic Local Alignment Search Tool (BLAST) on the NCBI platform to compare the obtained query sequences with reference sequences available in the GenBank database [36]. The BLAST analysis results indicated a high level of nucleotide similarity between the *plnA* gene sequences of *L. casei* and *L. paracasei* and those previously deposited in GenBank. However, the sequences obtained in this study represent partial gene fragments rather than full-length *plnA* genes, due to the primer design. The primers were specifically designed to amplify short regions of the target gene, similar to the DNA barcoding method—an approach for species identification using short, unique DNA sequences of a particular gene [35]. This method is intended to facilitate rapid and accurate identification and authentication of living organisms, with target sequence lengths typically ranging from 300 to 600 base pairs. DNA barcoding functions similarly to product barcodes, wherein each species possesses a unique "genetic code" that distinguishes it from others. In addition to the BLAST analysis, the nucleotide sequences generated in this study were also deposited in the GenBank database, with accession numbers OP873114 for *L. casei* and OP873115 for *L. paracasei* (Figure 2).

>OP873114 [organism=*Lactobacillus casei*] [isolate=NKY1] Plantaricin A (plnA) gene, partial cds
TAAAATGTACGTTAATAGAAATAATTTCCTCCGTACTTCAAAAACACA
TTATCCTAAAAGCGAGGTGATTATTATGAAAATTCAAATTAAAGGTAT
GAAGCAACTTAGTAATAAGGAAATGCAAAAAATAGTAGGTGGAAAGA
GTAGTGCGTATTCTTTGCAGATGGGGGCAACTGCAATTAAACAGGTAA
AGAAACTGTTTAAAAAATGGGGATGGTAATTGATTTA
>OP873115 [organism=*Lactobacillus paracasei*] [isolate=NKY2] Plantaricin A (plnA) gene, partial cds
ATTTCATGGTGATTCACGTTTAAATTTAAAAAATGTACGTTAATAGAA
ATAATTCCTCCGTACTTCAAAAACACATTATCCTAAAAGCGAGGTGAT
TATTATGAAAATTCAAATTAAAGGTATGAAGCAACTTAGTAATAAGGA
AATGCAAAAAATAGTAGGTGGAAAGAGTAGTGCGTATTCTTTGCA
GATGGGGGCAACTGCAATTAAACAGGTAAAGAAACTGTTTAAA
AAATGGGGATGGTAATTGATTTA

Figure 2. Sequencing results of the *plnA* gene in *L. casei* and *L. paracasei*.

Based on the results of BLAST analysis, it can be seen that the plnA gene sequences in *L. casei* and *L. paracasei* are homologous with the plnA gene sequence of *L. plantarum* (Table 1). The plnA gene sequence in *L. casei* has a similarity with the plnA gene sequence of *L. plantarum* strain EG.LP.18.7 by 99.56%. At the same time, the plnA gene sequence of *L.*

paracasei has similarities with the plnA gene sequence of *L. plantarum* strain EG.LP.18.7 by 100%. According to Ilyanie *et al*., maximum identity is the highest value of the percentage of identity or match between query sequences and sequences in the database [35]. The query sequence is declared a match if the similarity percentage is not less than 97%.

Table 1. BLAST results of *plnA* gene sequences of *L. casei* and *L. Paracasei.*

Spesies	BLAST Results		
	Homologous species	Ident (%)	Seq Id
L. casei OP873114	*L. plantarum* strain EG.LP.18.7 plantaricin A (plnA) gene	99,56	MN172266.1
L. paracasei OP873115	*L. plantarum* strain EG.LP.18.7 plantaricin A (plnA) gene	100	MN172266.1

This study shows the presence of the plnA gene in *L. casei* and *L. paracasei*, which has similarities with the plnA gene in *L. plantarum*. It is widely reported that plnA activity is well known as a pheromone peptide that works in inducing plantaricin bacteriocin production in *L. plantarum*. According to Stoyancheva *et al*., there are several peptides that are functionally similar to plnA, which have been detected in other bacteriocin-producing lactic acid bacteria [36]. In the research of Bu *et al*., it is mentioned that the plnA gene became the target of bacteriocin coding genes for detection in LAB, such as *Pediococcus pentosaceus*, *L. plantarum*, *L. casei*, *Lactobacillus sakei*, and *Lactobacillus viridescens* [27]. The results shown in the research of El Issaoui *et al*. show that the plnA gene was found in *L. plantarum 11* and *Weissella paramesenteroides* 36 [20].

Bacteriocins are created due to the presence of genes encoding bacteriocin production that are organized in operons. Genetically, the operon is made up of structural (bacteriocin production), immunity, and secretion genes. According to Bu *et al*., the mechanism of bacteriocin production from some LAB involves pheromone peptides in some circumstances, such as plantaricin production by *L. plantarum* and *gasserin* production by *L. gasseri* [37]. According to Jabbar *et al*., the presence of the plnA gene is a common feature of LAB that includes pheromone peptides in its bacteriocin production [38]. Because the plnA gene produces pheromone peptides that induce the transcription of genes arranged in an operon, the presence of pheromone peptide-coding genes in Lactobacillus can be associated with the production of bacteriocin, which has a similar biosynthetic process. The pheromone peptide is found in the quorum sensing mechanism, a system of signaling that activates bacteriocin synthesis. PlnA pheromone peptide activity in bacteriocin production is linked to histidine kinase. The pheromone peptide will bind to histidine kinase, causing autophosphorylation and subsequent phosphate group transfer to the regulatory response. The regulatory response will attach to specific promoter elements, causing gene expression to be activated [39].

According to Jabbar *et al*., partial characterization screening of the purified *L. plantarum* bacteriocin falls into the class II bacteriocin category [38]. Bacteriocins classified as Class II have a molecular weight of <10 kDa, broad inhibitory activity, are heat stable, and have an acidic to alkaline pH [14]. The similarity of the plnA gene expressing bacteriocin in *L. plantarum* to *L. casei* and *L. paracasei* has to be investigated further. These properties can be utilized to determine the similarities and differences between the bacteriocins produced, allowing for the development of their potential.

4. Conclusions

The plnA gene encoding bacteriocin can be detected in two *L. casei* species, *L. casei* and *L. paracasei*. This suggests that the antibacterial activity produced by *L. casei* and *L.*

paracasei is not only derived from the production of organic acids (lactic acid) but also from the production of bacteriocins. Based on the sequence analysis of the plnA gene from *L. casei* and *L. paracasei*, there is a similarity with the plnA gene from *L. plantarum* strain EG.LP.18.7. These findings indicate the potential for broader application of *L. casei* and *L. paracasei* in food preservation through bacteriocin-mediated antimicrobial activity.

Author Contributions

Conceptualization, N.K. and U.U.; methodology and software, N.K.; validation, U.U., J.K., and N.K.; formal analysis, S.S.; investigation, U.U.; resources, J.K.; data curation, J.K.; writing—original draft preparation, N.K; writing—review and editing, S.S.; visualization, N.K.; supervision, U.U.; project administration, S.S.; funding acquisition, N.K. All authors have read and agreed to the published version of the manuscript.

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Informed Consent Statement

Not applicable.

Data Availability Statement

Data supporting the findings of this study are available upon reasonable request from the corresponding author.

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Conflicts of Interest

The authors declare no conflict of interest.

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