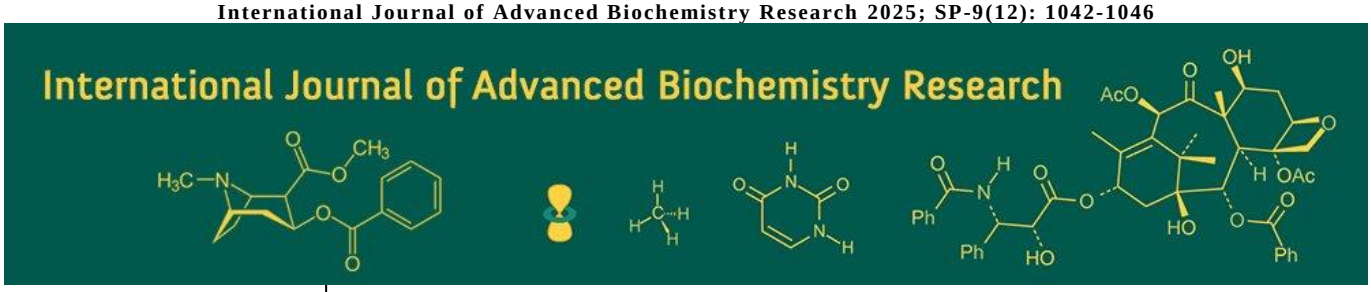

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Antibacterial activity and activation of plant immunity by *Bacillus velezensis* in tomato against bacterial leaf spot pathogen

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Abstract
Bacterial leaf spot of tomato, caused by *Xanthomonas euvesicatoria*, is associated with high genomic plasticity that enables rapid adaptation, virulence evolution, and the development of antibiotic resistance. These characteristics limit the effectiveness of conventional disease management strategies and underscore the need for sustainable alternatives to chemical control. This study evaluated the *In vitro* antibacterial activity of crude flagellin and exopolysaccharides (EPS) derived from *Bacillus velezensis* against *Xanthomonas euvesicatoria. It* investigated the molecular basis of flagellin recognition by the tomato FLAGELLIN-SENSING 2 (LeFLS2) receptor using computational methods. Antibacterial activity was measured using the poisoned food technique. Crude flagellin demonstrated the highest growth inhibition (31.11%), compared to EPS (15.56%) and *Bacillus velezensis* (16.67%). These results indicate that crude flagellin exhibits superior antibacterial activity. Sequence analysis of *B. velezensis* flagellin identified several conserved amino acid residues associated with flg22 epitope recognition, suggesting its ability to activate FLS2-mediated immune signaling in tomato. Molecular interaction analysis revealed a stable leFLS2-flagellin complex, supported by multiple hydrogen bonds and hydrophobic interactions, which facilitate adequate ligand accommodation within the receptor binding pocket. Docking analysis confirmed a strong and reliable interaction, with a docking score of −203.9 and a confidence score of 0.746. Collectively, these findings demonstrate the dual role of *B. velezensis* flagellin as both a direct antibacterial agent and an immune-eliciting molecule, highlighting its potential for eco-friendly disease management in tomato cultivation.

Keywords: Flagellin, Exopolysaccharide, Xanthomonas, tomato, molecular docking

Introduction
Plants possess an innate immune system that enables rapid detection and response to invading pathogens. Pattern-triggered immunity (PTI) is a central component of this system, activated when conserved pathogen-associated molecular patterns (PAMPs) are recognized by host pattern recognition receptors (PRRs) at the plasma membrane (Osdaghi et al., 2021) [1]. Bacterial flagellin, a highly conserved structural protein essential for bacterial motility and virulence, is among the most extensively studied PAMPs (Timilsina et al., 2020) [2]. Recognition of flagellin initiates early immune responses in plants, such as the production of reactive oxygen species, activation of mitogen-activated protein kinase cascades, and transcriptional reprogramming, which collectively restrict pathogen growth (Potnis, 2021) [3]. In plants, flagellin perception is mediated by FLAGELLIN-SENSING 2 (FLS2), a leucine-rich repeat receptor-like kinase (LRR-RLK). FLS2 specifically detects a conserved 22-amino acid epitope of flagellin, known as flg22, located in the protein's N-terminal region (Hind et al., 2016) [4]. Structural and functional studies in *Arabidopsis thaliana* have shown that flg22 binding induces conformational changes in FLS2, facilitates its association with the co-receptor BAK1, and activates downstream immune signaling pathways. These investigations have also identified critical residues within the FLS2 ectodomain necessary for ligand binding and receptor activation, thereby elucidating the mechanisms of flg22-mediated immune perception in *Arabidopsis*. Tomato (*Solanum lycopersicum*), an economically significant crop, encodes a functional FLS2 homolog (LeFLS2) that can recognize flagellin and activate PTI. Nevertheless, the molecular mechanisms underlying *Bacillus* flg22 recognition by tomato FLS2 are less well characterized compared

to those in *Arabidopsis*. Sequence divergence among plant species may affect ligand specificity, binding affinity, and immune responses, highlighting the importance of comparative and species-specific analyses to clarify the structural basis of flagellin perception in tomato.

Bacillus velezensis is a beneficial bacterium known for its antagonistic activity against a range of plant pathogens and its ability to promote plant health (Robatzek et al., 2007) [6]. The flagellin of *B. velezensis* is important not only for bacterial motility but also as a potential elicitor of plant immune responses. Understanding how plant PRRs, such as LeFLS2, recognize *B. velezensis* flagellin can improve knowledge of plant-microbe interactions and inform the development of novel disease management strategies (Mueller et al., 2012) [7]. In silico structural approaches offer an effective means to investigate receptor-ligand interactions, particularly when experimental structural data are limited. Protein-protein docking and interaction analyses can identify key binding residues, predict interaction stability, and provide mechanistic insights into immune recognition (Mohite et al., 2019) [8]. This study utilized a comprehensive computational approach to examine the interaction between *Bacillus velezensis* flagellin and tomato FLS2 (LeFLS2). By integrating sequence alignment, epitope identification, protein-protein docking, and interaction visualization, the study aimed to identify key LeFLS2 residues involved in flg22 recognition and characterize the molecular interface governing flagellin perception in tomato. The findings offer structural insights into flg22-mediated immune recognition in tomato and enhance understanding of both conserved and species-specific aspects of FLS2-dependent plant immunity.

2. Materials and Methods

2.1. *In vitro* Antibacterial Assay

The antibacterial efficacy of crude flagellin and exopolysaccharides (EPS) against *Xanthomonas euvesicatoria* was assessed *In vitro* using the poisoned food technique. The culture medium was supplemented with *Bacillus velezensis* and Bacillus-derived crude flagellin, and EPS, and medium containing only Xanthomonas served as the control. Antibacterial activity was quantified by measuring *Xanthomonas* growth, and inhibition was determined by comparing colony development between treated and control media. The percentage inhibition of growth was calculated as follows: Percent inhibition (%) = (C - T) / C × 100, where C represents Xanthomonas growth in the control and T represents growth in the treated medium.

2.2. Retrieval of Protein Sequences

The amino acid sequence of tomato Flagellin-Sensing 2 (LeFLS2) was obtained from the National Center for Biotechnology Information (NCBI) protein database (Accession No. XP_004233092.1). The FLS2 sequence from *Arabidopsis thaliana* was sourced from published structural studies. The flagellin sequence of *Bacillus velezensis* was selected for epitope identification and interaction analyses. The flg22 peptide sequence was retrieved from the Protein Data Bank (PDB ID: 4MNA, chain C).

2.2. Multiple Sequence Alignment and Identification of Key Residues:

Multiple sequence alignment of *Arabidopsis thaliana* FLS2 and tomato FLS2 (LeFLS2) was conducted using the alignment described by Sun et al. (2013). Conserved residues were highlighted in red, similar residues in yellow, and residues involved in flg22 recognition were marked with blue solid circles. This comparative analysis identified key amino acid residues in LeFLS2 that may be involved in flagellin or flg22 recognition. To define the minimal flagellin epitope, the flg22 peptide sequence was aligned with the full-length flagellin sequence of *Bacillus velezensis*. This alignment confirmed a conserved 22-amino-acid region in the N-terminal domain of flagellin, corresponding to the canonical flg22 epitope.

2.3. Protein-Protein Docking Analysis:

Protein-protein docking was conducted to investigate the molecular interaction between *Bacillus velezensis flagellin or flg22* and tomato FLAGELLIN-SENSING 2 (LeFLS2). Docking simulations were carried out using the HDOCK server, which integrates template-based modeling with free docking methods. Multiple docked complexes were generated for the ligand-receptor pair. The final docked model was selected based on the lowest docking score, highest confidence score, and an acceptable ligand root-mean-square deviation (RMSD), ensuring a reliable and biologically relevant representation of the flagellin-LeFLS2 interaction. The molecular interactions within the selected docked complex were analyzed and visualized using LigPlot+. Hydrogen bonds were identified based on geometric criteria and represented as green dashed lines with corresponding bond lengths. Hydrophobic interactions were visualized as red semicircular arcs surrounding the interacting residues. Amino acid residues participating in hydrogen bonding and hydrophobic interactions were identified, enabling detailed characterization of the molecular interface involved in flagellin recognition by LeFLS2. Docking metrics, including docking score, confidence score, and ligand RMSD, were used to assess the interaction strength and structural stability of the *Bacillus velezensis* flagellin-LeFLS2 complex. These parameters provided quantitative evidence for evaluating the reliability and consistency of the predicted receptor-ligand interaction.

3. Results

3.1. *In vitro* Antibacterial Activity of Crude Flagellin and EPS

The antibacterial efficacy of Bacillus, as well as Bacillus-derived crude flagellin and exopolysaccharides (EPS), against *Xanthomonas euvesicatoria* was evaluated using the poisoned food technique. All treatments resulted in a measurable reduction in pathogen growth compared to the untreated control. Crude flagellin exhibited the highest antibacterial activity, with 31.11% inhibition, indicating strong inhibitory potential against *Xanthomonas*. EPS and Bacillus treatments showed 15.56% and 16.67% inhibition, respectively. These results demonstrate that crude flagellin exhibits superior antibacterial activity *in vitro* compared to EPS and Bacillus, indicating its greater potential as an antibacterial agent against *Xanthomonas euvesicatoria*

3.2. Tomato FLS2 and Bacillus flagellin interaction

Analysis of the flagellin sequence from *Bacillus velezensis* identified several key amino acid residues involved in the recognition of the flg22 epitope by the tomato FLS2 receptor. The residues contributing to this interaction include G154, L150, V200, T222, Y274, R296, Y298, N297, H316, T342, H344, N393, V412, S414, F417, S437, and V483. These residues are distributed throughout the flagellin protein and are considered important for effective receptor binding and activation of FLS2-mediated immune signaling in tomato.

3.3. Hydrogen bonding and hydrophobic interactions between leFLS2 and flagellin

Molecular interaction analysis demonstrated that the flagellin peptide (Chain B) forms a stable binding interface with the tomato leFLS2 receptor (Chain A) through both hydrogen bonding and hydrophobic interactions. Hydrogen bonds were observed between specific flagellin and leFLS2 residues, including Ser156(B)-Gly431(A), Asn278(B)-Asn363(A), Ser275(B)-Asn387(A), Asn271(B)-Leu385(A), Ile147(B)-His411(A), Asp42(B)-His221(A), Arg285(B)-Ser315(A), and Arg14(B)-Glu171(A). Hydrophobic interactions further stabilized the complex, with leFLS2 residues such as Leu385(A), His411(A), Gly431(A), Asn363(A), Asn387(A), Ser315(A), His221(A), and Glu171(A) surrounding key flagellin residues including Ile147(B), Asp42(B), Arg285(B), Arg14(B), Ser156(B), Asn278(B), Ser275(B), and Asn271(B). Together, these interactions stabilize the flagellin peptide within the leFLS2 binding pocket and facilitate effective ligand recognition and immune signaling activation (Figure1)

3.4. Analysis of the leFLS2-flagellin complex

Docking analysis of the leFLS2-flagellin (leFLS2-flg) complex indicated a strong binding interaction, as evidenced by a docking score of −203.9. The predicted interaction yielded a confidence score of 0.746, supporting the reliability and stability of the docking pose. The ligand root mean square deviation (RMSD) was 120.9 Å, reflecting conformational variation during docking. Collectively, these docking metrics support a favorable interaction between leFLS2 and flagellin, consistent with effective receptor-ligand recognition.

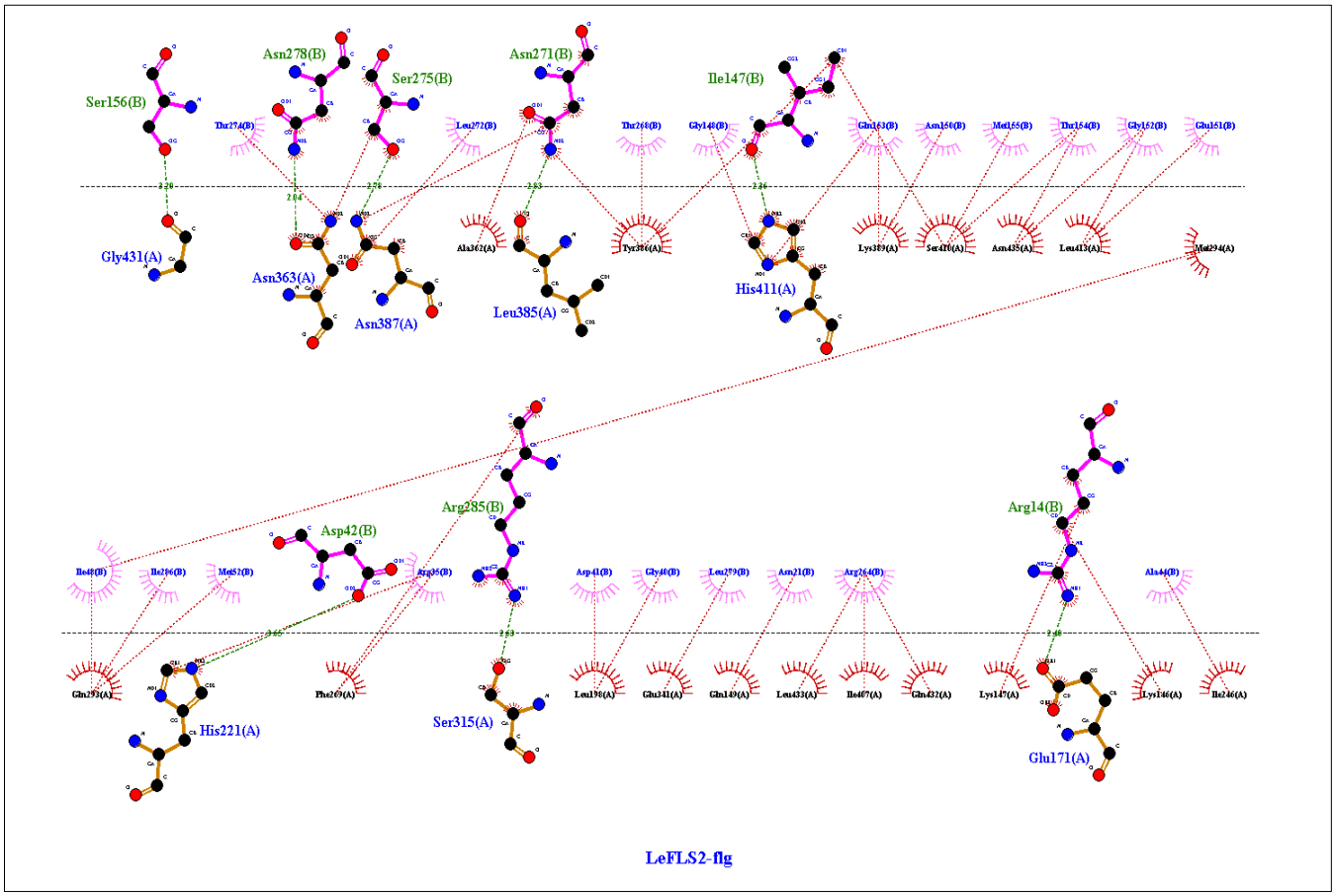

Fig 1: The interaction between tomato FLS2 (LeFLS2) and flagellin generated using LigPlot+. Hydrogen bonds are shown as green dashed lines with the corresponding bond lengths, while hydrophobic interactions are represented by red semicircles.

Discussion

Flagellin is a well-characterized microbe-associated molecular pattern (MAMP) that serves a dual function in plant-microbe interactions, acting both as an immune elicitor and direct antimicrobial agent (Robatzek et al., 2007; Mueller et al., 2012; Newman et al., 2013) [6, 7, 17]. This study integrates *In vitro* antibacterial assays with in silico molecular analyses to elucidate the antibacterial potential of *Bacillus velezensis*-derived flagellin and its recognition by the tomato FLAGELLIN-SENSING 2 (LeFLS2) receptor. The combined experimental and computational evidence offers complementary insights into the biological activity and immune relevance of *B. velezensis* flagellin, supporting its potential application in sustainable plant disease management. The poisoned food assay demonstrated that crude flagellin produced a significantly greater inhibitory effect against *Xanthomonas euvesicatoria* than exopolysaccharides (EPS) or *Bacillus*

velezensis treatments. This result is consistent with previous reports highlighting the sensitivity of Xanthomonas species to antimicrobial compounds from Bacillus spp. (Osdaghi et al., 2021; Potnis, 2021) [1, 3]. The pronounced growth inhibition observed with crude flagellin suggests that, beyond its role as a MAMP, flagellin may directly disrupt pathogen growth or cellular integrity. Similar antibacterial properties of bacterial proteins and peptides have been documented (Aslam et al., 2008; Mohite et al., 2019) [14, 8]. In contrast, EPS showed only moderate growth inhibition, suggesting lower antibacterial activity under *In vitro* conditions. This finding aligns with earlier studies reporting variable antimicrobial activity of EPS depending on composition and concentration (Vidhyalakshmi et al., 2016; Insulkar et al., 2018) [9, 10].

Sequence analysis at the molecular level identified several conserved residues in *Bacillus velezensis* flagellin that are involved in flg22 recognition, including G154, L150, V200, T222, Y274, R296, Y298, N297, H316, T342, H344, N393, V412, S414, F417, S437, and V483. The conservation of these residues supports the structural and functional maintenance of the flg22 epitope, a critical determinant of FLS2-mediated immune perception (Meindl et al., 2000) [5]. This observation is consistent with previous research demonstrating that conserved flg22 motifs are essential for effective recognition by plant pattern recognition receptors across diverse species (Hind et al., 2016) [4]. Molecular docking and interaction analyses confirmed the stability and specificity of the leFLS2-flagellin complex. The strong docking score (−203.9) and high confidence score (0.746) indicate a favorable and biologically relevant receptor-ligand interaction. The formation of multiple hydrogen bonds and extensive hydrophobic interactions between flagellin and leFLS2 residues highlights the importance of precise molecular complementarity for immune recognition. Comparable interaction networks have been reported in earlier structural and computational studies of FLS2-flg22 complexes (Hind et al., 2016; Banerjee et al., 2022) [4, 11]. The relatively high ligand RMSD observed in the docking analysis likely reflects the conformational flexibility of the flagellin peptide, a characteristic commonly associated with microbe-associated molecular patterns (MAMPs) that facilitates effective receptor engagement under varying physiological conditions (Algar et al., 2014; Castro et al., 2021) [16, 15]. The efficient interaction between B. velezensis flagellin and LeFLS2 underscores its potential role in triggering pattern-triggered immunity (PTI) in tomato. Activation of FLS2-mediated signaling is associated with rapid defense responses, such as reactive oxygen species production and the expression of defense-related genes, which ultimately contribute to disease resistance (Robatzek et al., 2007) [6]. The dual functionality of flagellin observed in this study, encompassing both direct antibacterial activity and immune elicitation, supports its potential application as a biocontrol component. These findings are consistent with previous studies that emphasize the significance of Bacillus spp. as effective biocontrol agents and the central role of flagellin in plant immune activation (Kakkar et al., 2015; Insulkar et al., 2018) [12, 10].

Conclusion

This study clarifies the molecular basis of flagellin perception by the tomato pattern recognition receptor LeFLS2 and demonstrates a strong, stable interaction with flagellin derived from Bacillus velezensis. The identification of conserved residues, as well as key hydrogen-bond and hydrophobic interactions, provides structural insights into receptor-ligand recognition and lays the foundation for future research on effector-mediated immune evasion in bacterial pathogens. Beyond its immune-eliciting function, *B. velezensis* flagellin displayed significant antibacterial activity against *Xanthomonas*. This dual functionality positions flagellin as both a direct antimicrobial agent and an effective inducer of plant defense responses. Collectively, these results underscore the potential of *B. velezensis* flagellin as a component of eco-friendly and sustainable disease management strategies. Additional in planta and field-level studies are required to confirm the protective efficacy of *B. velezensis* flagellin and to elucidate its role in activating downstream defense signaling pathways under natural conditions.

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