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Evaluation of antimicrobial action of probiotics against *Xanthomonas axonopodis* pv. *punicae* causing bacterial blight of *Punica granatum* L. (Pomegranate)

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ABSTRACT

The pomegranate is an economically important fruit crop in India that belongs to the family Punicaceae. It is considered a 'fruit of paradise' due to having higher nutritional and medicinal properties. Recently production of pomegranate yield has been lowered due to the bacterial blight of pomegranate. The bacterial blight of pomegranate is caused by Xanthomonas axonopodis pv. punicae. The currently available disease management strategies have failed, alarming the situation with the loss of the economy in India. Probiotics are the live, viable, and active microorganisms predominantly present in various fermented food products, that provide good health benefits and keep an active and balanced intestinal flora of essential microorganisms. This research article is based on the study of the antimicrobial action of probiotics against bacterial blight disease. The isolated strains from the pomegranate fruits and leaves were characterized by cultural morphological, biochemical, and molecular methods by 16S rRNA gene sequencing. The probiotics were isolated from various traditional fermented food products, including curd, idli batter, pickle, and jalebi batter, and were then characterized and identified as Lactobacillus spp. and Streptococcus spp. Different isolated probiotic strains were evaluated as antimicrobial compounds by agar well diffusion method. The zone of inhibition was observed using controls. The antimicrobial activity of probiotics against bacterial blight in pomegranates was studied and confirmed. Additionally, the probiotic strains were further tested for their antimicrobial activity in a pomegranate crop field, where significant results were observed.

Keywords: Antimicrobial activity, Bacterial blight, Pomegranate, Probiotics, *Xanthomonas axonopodis* pv. *punicae,* 16srRNA gene sequencing

INTRODUCTION

Pomegranate (*Punica granatum L.)* is a major economic fruit crop grown in India, and it is considered a "Vital Cash Crop" for farmers. Pomegranate is cultivated all over the world viz. Egypt, Morocco, Afghanistan, India, China, Japan, and the USA (California)(Kumar, 2018). India plays a big role in cultivating pomegranate and makes up half of the pomegranate production. Currently, the cultivation of pomegranates is increasingly challenging, with farmers facing numerous problems, particularly in major pomegranate-growing states in India (K.K. Sharma *et al.,* 2011). Bacterial blight of pomegranate, caused by *Xanthomonas axonopodis* pv. *punicae* was first reported by Hinorani and Mehta in 1952 and was subsequently studied by Hingorani and Singh in 1960 in India (S. B. Jagdale *et al.,* 2018). The high bacterial blight disease severity reported a 70-80 percent loss of pomegranate yield in major cultivating states of India (Rattan Bhardwaj *et al.,* 2014). The current disease management practices for the control include uprooting, burying, de-budding, blazing infected plant parts, disinfecting the crop cultivating tools, and spraying copper-based pesticides and streptomycin antibiotics (Nakayinga *et al.,* 2021). All available management strategies to control the bacterial blight disease pomegranate have failed in the complete eradication of pathogens (S. Jagdale & Kapadnis, 2021). Probiotics are 'live and active microorganisms' that have a lot of health-related benefits when administered in enough amounts. The administered probiotics result in the prevention of several physical conditions and diseases, as well as the prevention and treatment of others (Sadeghi *et al.,* 2022). The most probiotics are lactic acid bacteria (LAB) and they are present in yogurt, other fermented foods, and dietary supplement products. Several health benefits have been observed after the consumption of probiotics

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infection, and they tolerate bile salts and acidity of the stomach (*Karimi et al.,* 2018). Probiotics predominantly remain as part of the intestinal gut microflora, where they produce various antimicrobial substances such as lactic acid, bacteriocins, and H_2O_2, creating unfavorable conditions for pathogens and leading to an increased immune response in the host (Karimi *et al.,* 2018). Bacteriocins, which are small, heat-stable peptides composed of positively charged, hydrophobic amino acids, work in conjunction with organic acids to create an environment that is hostile to pathogenic microorganisms, thereby inhibiting their proliferation.

The health benefits have been mostly studied in specific probiotics of the following genera: *Lactobacillus, Bifidobacterium, Saccharomyces, Enterococcus, Streptococcus, Leuconostoc, Bacillus, Escherichia coli* and *Micrococcus* (Fijan, 2014). In this study, the probiotics were isolated from traditional fermented food products especially, curd, pickle, jalebi batter, and idli batter further isolates were characterized by morphological, cultural, and biochemical tests. Isolates strains belonged to the genera of *Lactobacillus* and *Streptococcus*. These strains were evaluated as antimicrobial compounds against bacterial blight by agar well diffusion method (Tajehmiri & Darsanaki, 2014). Zones of inhibition of selected probiotics were assessed against pathogens, and the effectiveness of these probiotics was further evaluated for antimicrobial activity in a pomegranate crop field.

MATERIAL AND METHODS

Isolation of *Xanthomonas axonopodis* pv. *punicae*

The samples exhibited characteristic symptomatic lesions on fruits and leaves, appearing as small dark brownish-black water-soaked spots (K.K. Sharma *et al.,* 2011). Collected pomegranate samples from various regions in the Solapur district showed infected fruits and leaves, which were washed twice in sterile distilled water. The ooze test was performed to confirm the presence of pathogens. The bits were treated with 0.1% $HgCl_2$ for 7-8 minutes and then further washed with sterile distilled water (Shali *et al.,* 2018). A loopful of crushed bits in sterile saline was inoculated onto sterile Nutrient Glucose Agar (NGA) plates and incubated at 28°C for 48 hours.

Characterization of isolates

Isolated colonies were characterized based on the morphological and cultural characteristics including size, shape, color, elevation, margin, opacity, pigmentation, and texture of the colony. A pure culture was prepared and Gram staining and motility tests were performed. Further, biochemical tests of pure cultures were performed, including the KOH string test, asparagine medium test, starch hydrolysis test, H_2S production test, gelatin liquefaction test, IMViC tests, catalase production test, oxidase test, urease test, caseinase test, sugar fermentation test, phenylalanine deamination test, nitrate reduction test, and Triphenyl Tetrazolium Chloride (TTZ) test (P. B. Kale, 2012). Additionally, these isolates were characterized using 16S rRNA gene sequencing (Basavand *et al.,* 2022).

Pathogenicity study (Koch's Postulates)

Isolated strains were tested for proving pathogens by detached leaf assay (Ramesh Ippikoppa, et.al. 2017). In this test, middle-aged pomegranate healthy leaves were selected and detached from the plants. Further, all leaves were washed with sterile distilled water, wiped with 70 percent absolute alcohol, and air dried. Prepared the inoculum of *Xanthomonas axonopodis* pv. *punicae* with 10^8 CFU/ml, sprayed over the dried pomegranate leaves, and one set was kept as a control without spraying. All leaves were incubated in sterile moistened plates at room temperature in an undisturbed place for 4-6 days. Observed the lesions on leaves after incubation and recorded the results compared with control.

Isolation and characterization of probiotics

For the isolation of probiotics, various fermented food products were selected, including curd, pickle, jalebi batter, and idli batter. Serial dilutions of the samples were prepared in sterile distilled water, ranging from 10^{-1} to 10^{-5}. Aliquots of 0.1 ml from the desired dilutions (10^{-3}, 10^{-4}, and 10^{-5}) were inoculated onto MRS agar plates (De Man, Rogosa, and

Sharpe agar, which is specifically selective for the growth of *Lactobacilli* spp.) and incubated at 37°C for 24 hours (Morsaline Billah *et al.,* 2010). The isolated strains were further characterized using morphological, cultural, and biochemical tests, including sugar fermentation tests, IMViC tests, oxidase test, catalase test, caseinase test, acid tolerance test, H_2S test, and arginine hydrolysis test.

Antimicrobial activity of probiotics

The characterized strains were confirmed and then used for the enrichment and mass production of probiotic cultures for further antimicrobial testing. The mass-produced probiotic culture was tested against the isolated strains using the agar well diffusion method (Morsaline Billah *et al.,* 2010). MRS medium was prepared with both hard basal agar and soft agar. The probiotic culture was centrifuged at 12,000 rpm for 15 minutes. The pellet was discarded, and the supernatant was used for antimicrobial testing. Soft agar tubes were inoculated with *Xanthomonas axonopodis* pv. *punicae* culture and poured onto the hard basal agar plates, allowing the plates to solidify. Wells were prepared in the solidified plates, and 0.1 ml of probiotic supernatant was added to the wells, with test controls (distilled water) included. The plates were incubated at 28°C for 24-48 hours, and inhibition zones were observed and recorded. This experiment was conducted in triplicate.

Evaluation of probiotics as antimicrobial

For this experiment, probiotic culture supernatant was prepared as described previously. The experiment was conducted in a pomegranate crop field. The cell-free supernatant was formulated in a sterile phosphate buffer at pH 6.8 and then sprayed onto infected pomegranate fruits in the field. The treated infected fruits were labeled as the test group, while untreated infected fruits served as controls (Hamed *et al.,* 2011). The plants were covered with polythene sheeting to prevent contamination from external sources. After 7-8 days, symptomatic lesions were observed periodically, and the results were recorded (Chavan *et al.,* 2016).

RESULTS

In the morphological and cultural study, four well-isolated colonies were selected on Nutrient Glucose Agar (NGA) plates and designated as Xap-I, Xap-II, Xap-III, and Xap-IV. The colonies exhibited pale yellow coloration, mucoid texture, slight elevation, round shape, and opacity, and were Gram-negative rods with motility. The KOH string test was positive, indicating a viscous mixture. Detailed morphological characteristics are provided in Table 1.

Table 1: Morphological and cultural characteristics of isolates

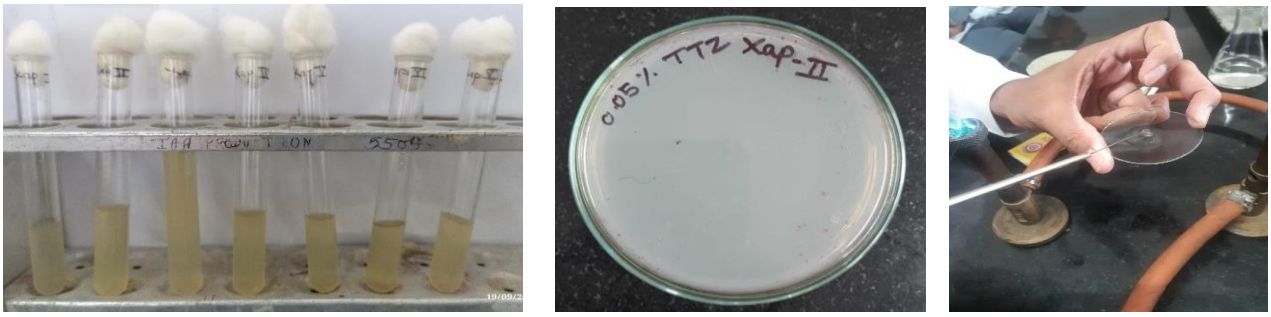
Isolate	Size	Shape	Color	Margin	Elevation	Opacity	Consistency	Gram nature	Motility
Xap I	1 mm	Circular	Pale yellow	Entire	Raised	Opaque	Mucoid	Gram-negative	Motile
Xap II	2 mm	Circular	Pale yellow	Entire	Raised	Opaque	Mucoid	Gram-negative	Motile
Xap III	1 mm	Circular	Pale yellow	Entire	Raised	Opaque	Mucoid	Gram-negative	Motile
Xap IV	1 mm	Circular	Pale yellow	Entire	Raised	Opaque	Mucoid	Gram-negative	Motile

In the biochemical characterization, the isolated strains showed positive results for glucose and lactose utilization, H_2S production, gelatin hydrolysis, caseinase, oxidase, catalase production, starch hydrolysis, and phenylalanine deamination. A low concentration of Triphenyl Tetrazolium Chloride (TTZ) inhibited the complete growth of *Xanthomonas,* confirming the presence of *Xanthomonas spp.* Negative results were observed for the IMViC, lysine utilization, nitrate reduction, and asparagine medium test where asparagine serves as the carbon as well as nitrogen source, these results are indicative of *Xanthomonas spp*. (P. B. Kale, 2012). The isolates were identified as *Xanthomonas axonopodis* pv. *punicae*. Detailed biochemical characteristics are presented in Table 2, with images shown in Figure 1.

Table 2: Biochemical characters of isolates

Sr. No.	Biochemical Test	Xap I	Xap II	Xap III	Xap IV
1.	Indole test	-	-	-	-
2.	Methyl Red Test	-	-	-	-
3.	Voges Proskauer Test	-	-	-	-
4.	Citrate Test	-	-	-	-
5.	Asparagine Medium Test	-	-	-	-
6.	Lysine Utilization Test	-	-	-	-
7.	H_2S Test	-	-	-	-
8.	Nitrate Reduction Test	-	-	-	-
9.	Urease Test	-	-	-	-
10.	Gelatine Hydrolysis Test	+	+	+	+
11.	Oxidase Test	+	+	+	+
12.	Catalase Test	+	+	+	+
13.	Starch Hydrolysis Test	+	+	+	+
14.	Phenylalanine Deamination	+	+	+	+
15.	Caseinase Test	+	+	+	+

'+'indicative isolated organisms able to produce/utilize, '-'Indicative organisms unable to produce/utilize



a) b) c)

d) e) f)

a).Gelatine liquefaction test, b) TTZ Test, c) KOH string test d) Starch hydrolysis, e) Oxidase test, and f) Catalase test

Fig. 1: Biochemical characterization of *Xanthomonas axonopodis* pv. *Punicae*

Molecular characterization was performed using 16S rRNA gene sequencing, the target gene was the bacterial 16S rRNA. Amplification was carried out using universal primers 16S27F and 16S1492R, and PCR products were purified using salt purification (Weisburg *et al.,* 1991). Agarose gel electrophoresis was conducted to assess PCR amplicon quality, and purified amplicons were subjected to cycle sequencing with BDT v3.1 chemistry and sequenced on an ABI 3500XL Genetic Analyzer. Additional internal primers were used to obtain near-full-length sequences. Trace files were manually reviewed, curated, and converted into FASTA format. The consensus sequence was searched against the SILVA database v138.1 using the BLAST tool (Quast *et al.,* 2013). BLAST results are shown in Table 3.

Table 3: BLAST results of isolates

Sr. No.	HM Code	Sample ID	Result: - Closest Taxonomic Relative
1	23H110 289	Xap I	*Xanthomonas axonopodis* pv. punicae (CAGJ01000132)
2	23H110 290	Xap II	*Xanthomonas axonopodis* pv. punicae (CAGJ01000132)

For the phylogenetic analysis, up to 10 closest-neighbor sequences belonging to different taxa from amongst the top 1000 hits with the highest similarity in the search results were retrieved from the database and aligned using the MUSCLE aligner (Edgar, 2004). The multiple sequence alignment was manually inspected and used to construct a consensus phylogram using a maximum likelihood algorithm with 1000 iterations using MEGA 11 software (Tamura *et al.,* 2021). Based on the phylogenetic arrangement, isolate 23H110_289_Xap I showed a close evolutionary relationship with *Xanthomonas axonopodis* (CAGJ01000132), and isolate 23H110_290_Xap_II showed a close evolutionary relationship with *Xanthomonas axonopodis* (CAGJ01000132), confirming the isolates as *Xanthomonas axonopodis* pv. *punicae.* The phylogenetic analysis is shown in Fig. 2. and Fig. 3.

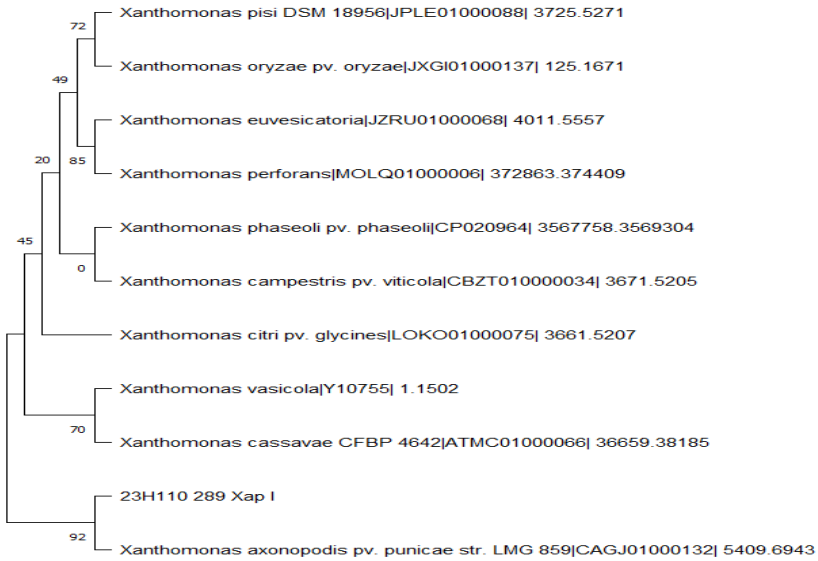


Fig. 2: Phylogenetic analysis of Xap I

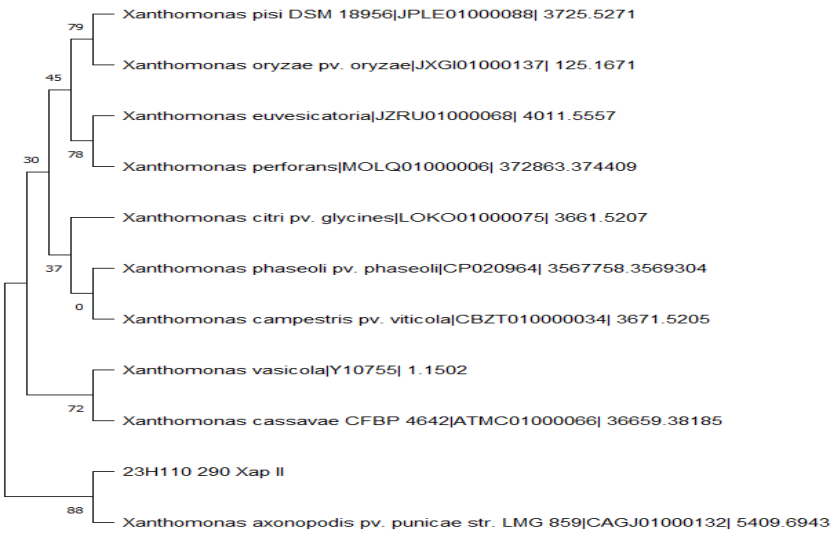


Fig. 3: Phylogenetic analysis of Xap II

Pathogenicity was assessed using Koch's Postulates through a detached leaf assay (Ramesh Ippikoppa et.al., 2017). Black-brown lesions developed on leaves incubated on sterile moistened plates at room temperature for 4-6 days. Lesions were compared with control leaves. The pathogenicity of the isolates was confirmed as they were able to cause bacterial blight disease on pomegranate (S. B. Jagdale *et al.,* 2018), fulfilling Kotch's postulates. Results are shown in Fig. 4.

a) Inoculated leaves b) Control leaves without inoculation

Fig. 4: Pathogenicity test by detached leaf assay

Probiotics were isolated from traditional fermented products, including curd, pickle, jalebi batter, and idli batter. After 24-48 hours of incubation, colonies were small to medium-sized, white-colored, circular, raised, moist, and opaque on MRS agar, and were gram-positive rods or cocci with motile cells. Morphological and cultural characteristics are detailed in Table 4.

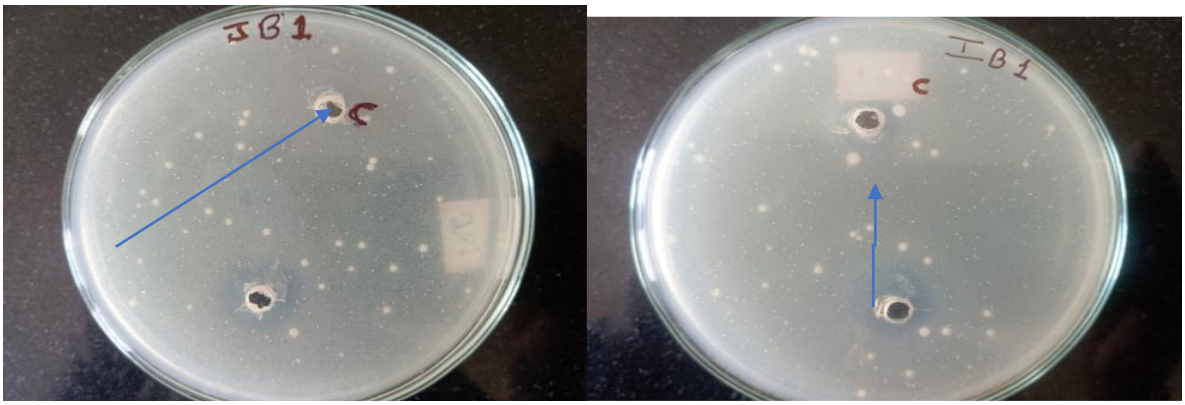
Biochemical tests showed positive results for sugar fermentation, IMViC, oxidase, catalase, caseinase, acid tolerance, H_2S production, and arginine hydrolysis. Four isolates were identified as *Lactobacillus* spp. and two as *Streptococcus* spp. These strains were tested for antimicrobial activity against *Xanthomonas* strains.

Table 4: Morphological and cultural characteristics of probiotics

Isolate	Size	Shape	Color	Margin	Elevation	Opacity	Consistency	Gram nature	Motility
JB I	3 mm	Circular	White	Regular	Raised	Opaque	Moist	+ ve	Non-motile
JB II	3 mm	Circular	White	Regular	Raised	Opaque	Moist	+ ve	Non-motile
JB III	3 mm	Circular	White	Regular	Raised	Opaque	Moist	+ ve	Non-motile
JB IV	3 mm	Circular	White	Regular	Raised	Opaque	Moist	+ ve	Non-motile
IB I	1 mm	Circular	White	Regular	Flat	Opaque	Moist	+ ve	Non-motile
IB II	1 mm	Circular	White	Regular	Flat	Opaque	Moist	+ ve	Non-motile

Note- JB- Jalebi batter, IB- Idli batter

The antimicrobial activity of the isolated probiotics was evaluated by measuring the zones of inhibition on agar plates. After incubation, zones of inhibition measuring approximately 16-18 mm were observed (Figure 5), indicating significant antimicrobial activity. Although the inhibition zones were not clearly defined, *Xanthomonas* spp. growth was notably inhibited, confirming the probiotics' antimicrobial potential. These results were extended to an in vivo crop field assay, where a significant halt in the infection was observed on infected pomegranate plants treated with probiotics and protected by polythene sheeting. After 7-8 days, the spread of symptoms was effectively halted, and a notable reduction in fruit lesions was recorded. These findings confirm that probiotics possess antimicrobial activity against *Xanthomonas axonopodis* pv. *punicae*, the causative agent of bacterial blight in pomegranate.



JB=Jalebi Batter, IB= Idli Batter, and C= Control

Fig.5: Antimicrobial action of isolated strains of probiotics against *Xanthomonas axonopodis* pv. *punicae* with control

DISCUSSION

The present study evaluates the antimicrobial efficacy of probiotics against *Xanthomonas axonopodis* pv. *punicae* (Xap), the pathogen responsible for bacterial blight in pomegranate. The results demonstrate that probiotics, specifically isolated from traditional Indian fermented foods, exhibit significant antimicrobial activity against Xap in vitro and in vivo. This finding presents a promising alternative to current ineffective management strategies for bacterial blight in pomegranate, which is a critical economic crop in India.

Antimicrobial Action of Probiotics

The probiotics isolated from fermented foods, identified as *Lactobacillus* spp. and *Streptococcus* spp., showed strong inhibitory effects against Xap in agar well diffusion assays. Zones of inhibition ranging from 16 to 18 mm were observed, indicating that these probiotics produce antimicrobial compounds capable of suppressing the growth of Xap. This antimicrobial action can be attributed to the production of organic acids, bacteriocins, and hydrogen peroxide by these probiotic strains, which create unfavorable conditions for the pathogen.

The observed antimicrobial activity aligns with previous research indicating that lactic acid bacteria, particularly *Lactobacillus* spp., produce bacteriocins and other antimicrobial substances that inhibit pathogenic bacteria. For instance, *Lactobacillus plantarum* has been shown to produce plantaricin, a bacteriocin effective against several Gram-positive and Gram-negative bacteria, which supports our findings of its effectiveness against Xap.

Previous research has consistently shown that various probiotic species exhibit antimicrobial activity against a range of human and plant pathogens. For instance, *Lactococcus lactis*, *Enterococcus faecium*, *Lactobacillus helveticus*, and *Streptococcus thermophilus* strains were evaluated for their effects on *Xanthomonas* spp., with findings indicating that both the isolated strains and their supernatants were inhibitory to *Xanthomonas* spp. (Mačionienė *et al.,* 2022). Additionally, probiotics have been investigated for their efficacy against plant pathogens such as *Rhizoctonia solani* and *Fusarium oxysporum*, which are common causative agents of root rot and wilt diseases in pepper plants (El-Kazzaz *et al.,* 2022)

In the context of this study, isolated lactic acid bacteria (LAB) strains, including *Lactococcus lactis* subsp. *cremoris* PB6, *Lactobacillus brevis* PFR 77, and *Lactococcus lactis* subsp. *cremoris* PFL9, were shown to be potent antagonists of *Xanthomonas axonopodis* pv. *punicae*, the pathogen responsible for bacterial blight in pomegranates (Gajbhiye, 2022). Moreover, probiotics have demonstrated substantial inhibitory effects on human pathogens such as *Listeria monocytogenes*, *Escherichia coli*, *Bacillus cereus*, and *Salmonella enteritidis* (Karimi *et al.,* 2018). These findings underscore the potential of probiotics as natural antimicrobial agents with broad applications in both agricultural and clinical settings.

Comparison with Current Management Strategies

Current strategies for managing bacterial blight of pomegranate include the use of copper-based pesticides and antibiotics like streptomycin. However, these methods have proven insufficient, primarily due to the development of resistance and the environmental impact of chemical treatments. The study's results suggest that probiotics could serve as a sustainable alternative, reducing the reliance on chemical controls. The field application of probiotic supernatants led to a significant reduction in lesion progression on infected fruits, supporting their potential use as biocontrol agents in pomegranate cultivation.

This finding is particularly relevant considering the growing concern over antibiotic resistance and the demand for organic agricultural practices. Probiotics, being natural and safe, fit well into the paradigm of sustainable agriculture and could be integrated into existing crop management practices with minimal environmental impact.

Limitations and Future Directions

Despite the promising results, there are limitations to this study that must be acknowledged. The in vitro assays, while indicative of antimicrobial potential, may not fully replicate the complex interactions that occur in a natural field environment. Factors such as environmental conditions, microbial community dynamics, and the stability of probiotic formulations under field conditions need further investigation.

Moreover, the study used a limited number of probiotic strains. Future research should explore a broader range of probiotics, including those from different sources, to identify the most effective strains against Xap. Additionally, the mechanisms underlying the observed antimicrobial effects should be elucidated through molecular studies, which could provide insights into optimizing probiotic formulations for field use.

Practical Implications and Broader Impact

The integration of probiotics into the management of bacterial blight in pomegranate could have significant practical implications. For farmers, this approach offers a cost-effective and environmentally friendly alternative to chemical treatments, potentially reducing crop losses and improving yield quality. Furthermore, the success of this approach in pomegranate could pave the way for the use of probiotics in managing other bacterial diseases in crops, thus contributing to the broader field of agricultural microbiology.

This study also contributes to the growing body of evidence supporting the use of probiotics beyond human health, extending their benefits to plant disease management. By harnessing the natural antimicrobial properties of probiotics, this research aligns with global efforts to promote sustainable agriculture and reduce the environmental footprint of crop production.

CONCLUSION

In conclusion, the study provides compelling evidence that probiotics isolated from traditional fermented foods in India can effectively inhibit *Xanthomonas axonopodis* pv. *punicae*, the causative agent of bacterial blight in pomegranate. The significant reduction in disease symptoms observed in both in vitro and field trials highlight the potential of probiotics as a viable alternative to chemical pesticides. However, further research is needed to optimize the application methods and explore the full range of probiotic strains available. With continued investigation, probiotics could become an integral part of sustainable disease management strategies in pomegranate and other economically important crops.

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