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ANTIFUNGAL ACTIVITY OF CERTAIN LACTIC STRAINS ISOLATED FROM RAW MILK AGAINST *PENICILLIUM GLABRUM*

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Abstract

Use of antifungal lactic bacteria and their bioactive metabolites is a good and sustainable biological way to fight fungi. In this study, 114 lactic strains isolated from raw milk of bovine, ovine, and caprine species were screened for their potential inhibitory activity against the fungus *Penicillium glabrum*. Among the isolated strains, 15 were found to exhibit antagonistic activity against the fungus in a direct in vitro confrontation test after incubation at 30°C for 48 hours. In the well diffusion method conducted using the supernatants of six isolates which showed strong inhibition, zones of inhibition around the wells, preventing the germination of *Penicillium glabrum* spores after incubation at 30°C for 48 hours, occurred in two of the supernatants. Inhibition was due to two lactic strains identified at the species level as *Leuconostoc mesenteroides* by 16S rRNA sequencing. These strains show a promising potential use in food bio-preservation.

Keywords: Raw milk, Lactic acid bacteria, Bioactive metabolites, Antifungal activity, *Penicillium glabrum*.

Introduction

Fungi, either mycotoxigenic or spoilage types, represent a serious issue for the agro-food industry (Davies et al., 2021; Pandey, Samota, Kumar, Silva, & Dubey, 2023; Wei et al., 2023). This occurs because huge economic losses are caused to different foodstuffs, and there is a high risk it presents to public health by consumption (Badiale Furlong et al., 2021; Y. Liu, Galani Yamdeu, Gong, & Orfila, 2020). Such fungi can be combated by various means, among them using chemical products; this has been associated with a number of disadvantages relating to public health, the environment, and production costs (Chen et al., 2023; Hirozawa, Ono, Suguiura, Bordini, & Ono, 2023; Maurya, Prasad, Das, & Dwivedy, 2021). Nowadays, consumers increasingly demand healthier foods with fewer chemical preservatives (A. Liu et al., 2022; Nasrollahzadeh, Mokhtari, Khomeiri, & Saris, 2022). To meet these demands and for economic and environmental reasons, players in the agro-food industry are turning to more environmentally friendly and ecologically sustainable biological preservation methods(Singh, Tiwari, & Dubey, 2021). The use of lactic acid bacteria with antifungal

activity is one of the bioconservation methods that has been pursued and supported in recent years due to the promising results they have shown and their biological nature, which is safe for consumer health (Bangar et al., 2021).

This work aimed to isolate lactic strains from raw milk of bovine, ovine, and caprine species and confront them with the fungus *Penicillium glabrum* in order to select and identify any lactic strains capable of inhibiting the growth of *P. glabrum*. Additionally, it aimed to identify the bioactive molecules synthesized by these strains.

Materials and Methods

This study discusses the experimental methodologies used to isolate and characterize lactic acid bacteria from raw milk, as well as test their antifungal efficacy against *Penicillium glabrum*. This section describes the procedures used to isolate the lactic strains, assess their antifungal capability, and identify the bioactive compounds that are responsible for this activity. The described techniques include the isolation of bacteria, direct confrontation tests with the target fungus, preparation of supernatants, as well as DNA extraction, amplification, and sequencing methods. These methods were chosen to allow a thorough evaluation of the effectiveness of the isolated lactic strains and to identify the molecular mechanisms involved in their antifungal activity. These steps are:

Isolation of Lactic Bacteria: The lactic strains originate from raw milk of bovine, ovine, and caprine species collected from various farms in northern Algeria. Appropriate dilutions were prepared using physiological saline. A volume of 100 µl from each dilution was inoculated into MRS and M17 culture media and incubated anaerobically at 30°C for 48 to 72 hours (de Man, Rogosa, & Sharpe, 1960; Panthavee, Pramuan, & Nasakom, 2007) .

Search for Antifungal Activity: The purpose of this study is to look into the potential antifungal properties of lactic isolates from raw milk. These isolates are tested in vitro against *Penicillium glabrum* using the double-layer streaking method on MRS medium. Lactic strains that showed substantial inhibitory action against the fungus were subsequently investigated using their supernatants to determine the capacity of these strains' bioactive metabolites to suppress the germination of *Penicillium glabrum* spores using the well diffusion method.

Direct Confrontation of Lactic Isolates with the Fungus: The search for lactic strains with antifungal activity was determined using the overlay method described by Magnusson and Schnürer (2001). Each lactic strain was streaked onto the surface of MRS agar medium in two parallel lines 2

cm apart, then incubated anaerobically at 30°C for 24 hours. The plate was then overlaid with malt extract agar (0.05% malt extract and 1% Oxoid agar) containing 10^5 spores/ml of the test fungus.

After 48 hours of incubation aerobically at 30°C, the inhibition zones were measured. The scale used was: (-) no visible inhibition, (+) fungal growth inhibition between 0.1% and 3% of the total plate area, (++) fungal growth inhibition between 3% and 8% of the total plate area, (+++) fungal growth inhibition greater than 8% of the total plate area.

Preparation of Supernatants: Each lactic isolate was inoculated into 100 ml of MRS broth at 30°C until an optical density (OD) of 540 nm = 2.6 was achieved. The acellular supernatant was prepared by centrifuging the broth at 11,500 g for 10 minutes, followed by sterilization through a sterile syringe filter with 0.45 µm pores (Muhialdin & Hassan, 2011). The pH was neutralized with 0.2 M phosphate buffer, and the effect of hydrogen peroxide was neutralized by adding catalase.

Inhibitory Activity of Bioactive Metabolites: The six lactic isolates that demonstrated strong inhibitory activity against *Penicillium glabrum* were further evaluated using the well diffusion method (Ivanova, Kabadjova, Pantev, Danova, & Dousset, 2000; Magnusson & Schnürer, 2001). Sterile potato dextrose agar (PDA) was inoculated with *Penicillium glabrum* spores at a concentration of 10^5 spores/ml. Fifteen milliliters of the inoculated medium were poured into sterile Petri dishes and allowed to cool to room temperature. Next, four wells, each 5 mm in diameter, were created using a sterile cork borer. Twenty microliters of sterile MRS agar were pipetted to cover the base of the wells to prevent leakage of the supernatant. Sixty microliters of the supernatant were pipetted into each well and allowed to diffuse for 30 minutes at 4°C, then incubated at 30°C for 48 hours. The diameter of the inhibition zone, expressed in millimeters, was determined using the following formula: (Diameter of the clear zone – Diameter of the well).

Protocol for Extraction, Amplification, and Sequencing:

DNA Extraction: DNA from the bacteria was extracted using the Nucleospin Kit from Macherey-Nagel, following the manufacturer's extraction procedure (Macherey-Nagel, Germany). The extracted DNA was quantified and assessed for quality using the NanoDrop 2000, evaluating the 260/280 and 260/230 wavelength ratios.

16S rDNA Amplification: Amplification was carried out using a P100 thermal cycler from Biorad (Biorad, USA). Universal primers (Nossa et al., 2010) were used:

- 63f (forward): 5' GAGTTTGATCMTGGCTCAG 3'
- 1492R (reverse): 5' GGTTACCTTGTTACGACTT 3'

The amplification products were visualized after electrophoresis on a 1.5% agarose gel with a 10 µl PCR product deposit, followed by staining in an ethidium bromide bath (0.5 µg/ml). After migration, the DNA was visualized and photographed under UV light.

PCR Product Purification: The PCR products were purified using the Clean Up Kit from Macherey-Nagel (MN., Germany), following the protocol described by the manufacturer.

Sequencing: The isolated and purified PCR products were sequenced using the Sanger technique (Sanger, Nicklen, & Coulson, 1977), employing the BigDye v3.1 kit from Applied Biosystems and the primers used for PCR amplification. The obtained sequences were analyzed and cleaned using the CHROMAS PRO software. The final sequences were compared with those in the GeneBank database using the BLAST program (https://blast.ncbi.nlm.nih.gov/Blast.cgiBlast) from NCBI to identify the studied isolates based on the percentage of homology with reference strains.

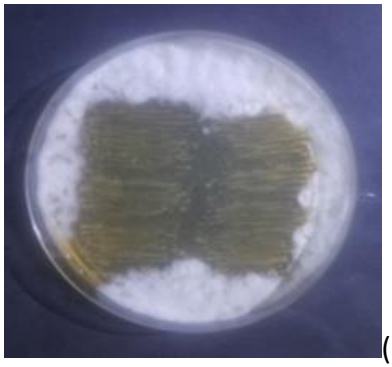
Results

Interest in using lactic acid bacteria as biocontrol agents has recently grown because of the fact that they have reportedly shown inhibition toward a very wide range of pathogenic microorganisms, including fungi. The capacity of lactic acid bacterial strains isolated from raw milk of different animal species (bovine, ovine, and caprine) to inhibit the growth of the fungus *Penicillium glabrum,* which is well known to contaminate different types of food products and cause relevant damages, was investigated.

Isolation and Characterization of Lactic Acid Bacteria: Phenotypic characterization of the isolated lactic acid bacteria was done on 114 lactic acid bacteria strains isolated from raw milk of the bovine, ovine, and caprine species.

Confrontation of Lactic Acid Bacteria Isolates with Fungi:

Out of the 114 lactic acid bacterial strains that were isolated and assessed for in vitro antifungal activity against *Penicillium glabrum*, using the overlay technique, 15 demonstrated antagonistic activity on the germination of such fungi. Of these 15 isolates, 6 lactic acid bacterial strains exhibited good, strong inhibitory activity.


(a)


(b)

Figure 1. Clear zones of growth inhibition of *Penicillium glabrum* around the streak lines of lactic acid bacteria (L31 & L33) after incubation at 30°C for 48h (a) and 72h (b) using the dual agar overlay method

Activity of Bioactive Metabolites: The 6 lactic acid bacterial isolates that exhibited strong inhibitory activity against *Penicillium glabrum* were further evaluated using the well diffusion method. The supernatants from 2 of these strains showed significant clear zones around the wells, indicating inhibition of *P. glabrum* germination, with relatively similar activity ranges. These strains are L31 and L33.

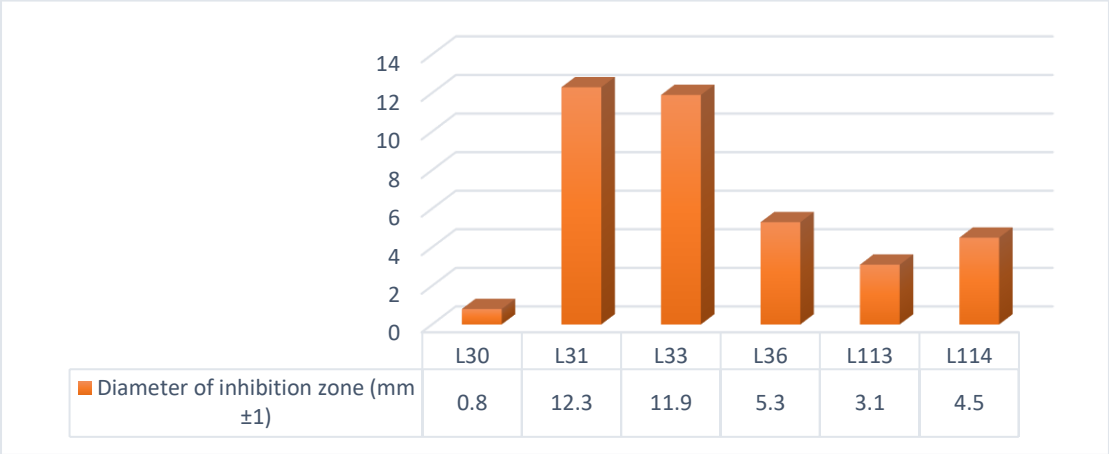


Figure 2. Diameters of inhibition zones around the wells

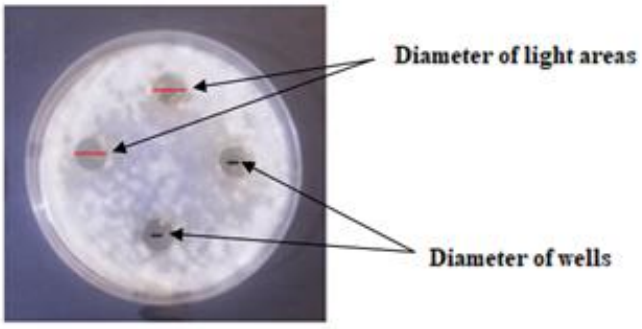


Figure 3. Clear zones around the wells corresponding to the inhibition of *Penicillium glabrum* following the diffusion of supernatant from strain L31 after 24 hours of incubation at 30°C.

Genomic Identification of Lactic Acid Bacteria Strains: Genomic analysis using 16S rRNA sequencing of the lactic acid bacterial strains exhibiting strong inhibitory activity revealed that the two strains, L31 and L33, are identified as Leuconostoc mesenteroides.

Discussion

These results, therefore, show the efficacy of the lactic acid bacteria isolated from raw milk against the common pathogenic fungus *Penicillium glabrum*. Of the 114 lactic acid bacterial strains tested for their antagonistic activity, 15 showed activity against *P. glabrum* germination, showing their huge potential for fungal control. Of these, 6 showed high inhibition.

Well diffusion method results obtained confirm that strains L31 and L33 form the largest inhibition zones; therefore, these strains are producing bioactive metabolites capable of efficiently inhibiting the growth of *P. glabrum*. The inhibition zones measured around the wells are an indication that Strains L31 and L33, among this studied collection, show very promising inhibition zones of about 12.3 mm and 11.9 mm in diameter, respectively. These results are consistent with observations made during the overlay tests, where these strains also showed strong inhibition of fungal growth.

Genomic characterisation of the lactic acid bacterial isolates showing high inhibitory activity identified them as *Leuconostoc mesenteroides*. This identification is important in working out the underlying mechanisms of the observed antagonistic activity and also for exploiting such strains in industrial or agricultural applications. In this regard, *Leuconostoc mesenteroides* is known to be a probiotic and produce different antimicrobial metabolites that could be the basis for such high activity against *P. glabrum.*

Conclusion

Out of the 114 lactic acid bacterial strains isolated from raw milk of bovine, ovine, and caprine species, 15 strains exhibited inhibitory activity against the fungus Penicillium glabrum. The inhibition potential varied among the strains. The 6 lactic acid bacterial strains that showed strong antagonistic activity were further studied, and among them, 2 strains yielded promising results and were identified as *Leuconostoc mesenteroides*. In conclusion, certain lactic acid bacterial strains from raw milk of bovine, ovine, and caprine species possess the ability to inhibit the growth of Penicillium glabrum. These strains hold potential for application in food preservation, pending further studies to fully understand their capabilities and optimize their use.

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