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Original Paper

Antifungal activity of lactic acid bacteria against *Fusarium* species responsible for tomato crown and root rots

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Abstract

Three lactic acid bacteria (LAB) (*Lactobacillus delbruckii* subsp. *bulgaricus, Leuconostoc mesenteroides* subsp. *dextranicum* and *Lactococcus lactis* subsp. *diacetylactis*) were tested for their antagonistic activity to reduce fungal growth of nine isolates of *Fusarium* species (*F. oxysporum, F. redolens* and *F. solani*), agents of tomato crown and root rot, the most destructive disease of tomato (*Solanum lycopersicum*) in Algeria. The LAB tested significantly inhibited nine strains of *Fusarium* spp. on Potato dextrose agar (PDA) and De Man, Rogosa, Sharpe agar (MRSA). Performance of *L. lactis* subsp. *diacetylactis* on MRS agar medium was 62.42% inhibition compared to 22.11% inhibition on PDA medium. Overlay assay showed that LAB secreted different metabolites, which inhibited growth of *Fusarium* spp. Inhibition by *L. mesenteroides* subsp. *dextranicum* was between 4.3 and 19.7% after 72 h incubation on PDA medium. Results showed that all LAB used can significantly reduce growth of various phytopathogenic *Fusarium* species, both by cell cultures and by their secondary metabolites.

Key words: antifungal activity, crown and root rot, *Fusarium* spp., lactic acid bacteria, tomato.
Abbreviations: LAB, lactic acid bacteria; Lmd, *Leuconostoc mesenteroides* subsp. *dextranicum*; Lld, *Lactococcus lactis* subsp. *diacetylactis*; Ldb, *Lactobacillus delbruckii* subsp. *bulgaricus*; MLM, Minimal Liquid Medium; MRSA, De Man, Rogosa, Sharpe agar; PDA, potato dextrose agar.

Introduction

Tomato (*Solanum lycopersicum* L.) is a socio-economically important crop in Algeria. In 2016, the production of tomato in Algeria reached 1 286 286 tonnes on a harvested area of 23 977 ha (FAO 2017). It is attacked by several species of *Fusarium*, of which there are two formae speciales of the morphospecies *Fusarium oxysporum*: *F. oxysporum* f. sp. *lycopersici*, responsible for vascular wilt disease (Geiser et al. 2004), and *F. oxysporum* f. sp. *radicis-lycopersici*, responsible for crown and root rot (Can et al. 2004). Three other species belonging to the same genus, *Fusarium commune, Fusarium redolens* (Hamini-Kadar et al. 2010) and *Fusarium equiseti* (Yezli et al. 2019) have also been reported as causal agents of crown and root rot of tomato. In addition, *Fusarium* species are reported not only in Algeria, but from different parts of the world, and they are known to be pathogenic to different plants (Mannai et al. 2018; Rezaee et al. 2018).

Several measures are used to control tomato diseases caused by *Fusarium* species, where the chemical fungicides are the most used, but they need to be replaced due to their harmful effect on the environment and human health (Bernard et al. 1997). Different alternatives strategies can be used, and biological control is the most important alternative (Karimi et al. 2012). In biological control, various methods can be used, inclusing essential oils (Hu et al. 2007; Bakkali et al. 2008; Lang, Buchbauer 2012; Yakoubi et al. 2019) and antagonistic microorganisms.

Important groups of microorganisms used in the biological control of fungal diseases are lactic acid bacteria (LAB) (Dalie et al. 2010; Laref, Guessas 2013; Zebboudj et al. 2014); plant growth promoting rhizobacteria (Nandhini et al. 2012; Abdulkareem et al. 2014; Yezli et al. 2015); and plant growth promoting fungi (Limón et al. 1999; Moreno-Maleos et al. 2007; Shentu et al. 2014). These microorganisms are ubiquitous, persistent in the environment, their isolation is simple and they are very variable, having specific and effective action (Karimi et al. 2012).

In Algeria, the average disease incidence caused by *Fusarium* species has reached 62% (Yezli et al. 2019), compared with 50% disease incidence caused by *Alternaria grandis* on the same culture (tomato) (Bessadat et al.

2016). This shows that there is not an effective treatment to reduce the disease incidence in Algeria. This raises interest in the potential effect of LAB, which are characterized by the secretion of different antimicrobial substances (Dalie et al. 2010). We previously tested the effect of LAB on *F. oxysporum* f. sp. *albedinis*, a causal agent of vascular fusariose of date palm, and found that these antagonistic bacteria can significantly reduce the growth of this phytopathogenic agent (Zebboudj et al. 2014).

The aim of the present study was to evaluate antifungal activity of three new strains of LAB isolated from camel milk against nine strains belonging to three pathogenic species of *Fusarium* on different culture media *in vitro* and *in vivo*.

Materials and methods

Fungal isolates

The fungal strains used in this assay were recovered from surface-sterilized root and stem base of diseased tomato plants using the method described by Leslie, Summerell (2006). Plants were collected from Mostaganem, Oran, Sekikda and Tlemcen departments in North Algeria. Single-spore isolates were compared with the original morphological species description of Nelson et al. (1983) and Leslie, Summerell (2006).

Molecular identification of fungal isolates

Molecular characterization was performed by sequencing the *Translation Elongation Factor 1-alpha* gene, using EF1 (5'-ATGGGTAAGGA(A/G)GACAAGAC-3') and EF2 (5'-GGA(G/A)GTACCAGT(G/C)ATCATGTT-3') primers recommended for *Fusarium* speices (O'Donnell et al. 1998). DNA was extracted using the method described by Edel et al. (2001). PCR was performed according to the following conditions: initial denaturation at 95 °C for 2 min; 34 cycles of denaturation at 95 °C for 30 sec, hybridization at 55 °C for 1 min, elongation at 72 °C for 90 sec; and final extension at 72 °C for 10 min. The resulting PCR products were sequenced and analyzed using the BLASTn program and compared to sequences in the GenBank nucleotide database. The nucleotide sequence data reported are available in the GenBank database (accession No. MG973088-MG973096).

Lactic acid bacteria strains

Three LAB strains isolated from camel milk were used in this study: *Lactobacillus delbruckii* subsp. *bulgaricus* (Ldb), *Leuconostoc mesenteroides* subsp. *dextranicum* (Lmd) and *Lactococcus lactis* subsp. *diacetylactis* (Lld). They were obtained from the Laboratory of Applied Microbiology collection, Faculty of Nature and Life Sciences, University Oran1 ABB, Algeria.

In vitro *assays: direct confrontation test*

This study was done using two culture media: Potato dextrose agar (PDA) and De Man, Rogosa, Sharpe agar (MRSA), to compare the antifungal activity on different substrates, using the method described by Gerbaldo et al. (2012) with a few modifications. LAB were seeded in two parallel ridges of 20 mm on both media. After incubation at 28 °C for 16h, a 5-mm fungal disc of each isolate with age 72 h was placed in the centre of culture media between the two ridges of LAB. Plates containing fungus alone were used as control and cultivated on both media. Incubation was done for 72 h at 28 °C. After incubation, fungal colony diameters were measured. Three replicates were used for each fungal isolate. The percentage of growth inhibition was determined using the formula described by Jafar-Pour et al. (2008).

In vitro *assays: overlay assay*

The overlay assay was performed according to the method described by Magnusson et al. (2003) with a few modifications. LAB were pre-cultivated in two parallel ridges of 20 mm on 10 ml of PDA at 28 °C for 12 h. After incubation, soft PDA with 0.7% agar was inoculated with 10^6 spores mL^{-1} of fungal suspension, homogenized and overlaid on pre-cultivated LAB cultures. The test was performed in triplicate. Inhibition areas around the bacterial ridges were measured after 72 h of incubation at 28 °C.

In vivo *assays: fungal pathogenicity tests*

A loam-sand mixture of 80:20 (vol/vol) was used. The loam soil was sifted to eliminate rocks and autoclaved at 121 °C for 90 min for three successive days (Trejo-Estrada et al. 1998). The pathogenicity of fungal isolates was confirmed according to the method described by Hamini-Kadar et al. (2010). For each fungal isolate, we used ten tomato seedlings of cultivar Montfavet 63-5 HF1 with age of twelve days. Roots were cut at 6 mm below the crown, and plants were placed in 10 mL Minimal Liquid Medium (MLM) inoculated with 10^6 spores mL^{-1}. After 10 min, the plants were transferred to the soil mixture. Infected plants were compared with positive controls using Fo47 (non-pathogenic strain) and strain NH48 (pathogenic strain) (Edel-Hermann et al. 2012) and with a negative control, prepared with MLM free fungal cells. Plants were placed in greenhouse conditions at 16/8 photoperiod, 28 ± 2 °C during the day, and 22 ± 2 at night. The plants were watered daily. Infection percentage was determined after three weeks.

In vivo *assays: antagonistic assays*

A LAB suspension of 10^6 cells mL^{-1} was prepared with physiological water from pre-cultivated cultures at 28 °C for 12 h, and poured onto the soil mixture around the crown 12 h before infection. Test plants were compared with infected plants of pathogenic tests as a positive control and with a negative control in which the LAB suspension

was replaced by sterilised physiological water. Plants were maintained in the same conditions of pathogenic tests but in separate growth rooms.

Statistical analysis

Fungal growth inhibition tests were subjected to analysis of variance (ANOVA) using R version 3.6.1. The means were compared at 5% level of significance and with Tukey HSD (Honestly Significant Difference) post-hoc tests.

Results

Morphological identification

Characterization on PDA medium showed a wide morphological variability with floccose, sparse or abundant mycelia. Colour varied between white to pale violet, pink or cream. Microscopic characteristics on Carnation Leaf Agar medium revealed 0 or 1 septate microconidia deposited on short or long monophialides forming false heads. Macroconidia were 3 to 7 septate. Terminal and intercalary chlamydospores were occurred singly or in pairs.

Molecular identification

Fungal isolates were identified at the species level using TEF-1α sequences. PCR products analyzed by the BLASTn program revealed that the isolates were *F. oxysporum* (Genbank accession No. MG973088, MG973090 and MG973096), *Fusarium solani* (Genbank accession No. MG973089, MG973093 and MG973094) and *F. redolens* (Genbank accession No. MG973091, MG973092 and MG973095), with similarity between 99.85 and 100% with MG599513, KF624788 and KT239482 GenBank sequences respectively.

Direct confrontation test

Results of direct confrontation between LAB cells and pathogenic fungus are presented by mycelia growth inhibition of *Fusarium* spp. on PDA and MRSA medium after 6 days of incubation (Fig. 1). All LAB used had fungal activity against *Fusarium* strains. Lld had a larger effect (significant) on MRSA medium, with 62.42% inhibition, followed by Ldb (60.68%) and Lmd (58.68%), compared to PDA medium, where there was 33.54 % inhibition by Lmd, followed by Ldb (28.32%) and Lld (22.11%) ($P < 0.05$). Total inhibition was between 55.95% against WY01 and 64.26% against WY18 on MRSA medium, compared to 22.56% against WY02 and 31.78% against WY18 on PDA medium. The results on Fig. 2 show that mycelium growth decreased significantly in presence of LAB cells, compared to control plates on both culture media, and more significant on MRSA medium ($P < 0.05$).

Overlay assay

The results of the overlay assay showed different effects of LAB on *Fusarium* growth (Table 1). Lmd had significant ($P < 0.05$) antifungal activity (between 4.3 and 19.7%), followed by Lld with significant ($P < 0.05$) inhibition (between 2.1 and 7.9%), and Ldb with only non-significant ($P > 0.05$) inhibition (2.1% after 48 h, decreasing to 1% on the third day of incubation on PDA medium). Strain WY18 was more sensitive to the action of all LAB used in this study. Strain WY03 was sensitive only to Lmd and resistant to the other LAB used. Figure 3 shows the growth inhibition of strain WY05 by LAB on PDA medium after 72 h incubation. Most importantly, inhibition differed with every fungal strain depending on the LAB used..

Fungal pathogenicity

The pathogenicity test showed that the nine fungal isolates used in this study were pathogenic and induced symptoms of crown and root rots, compared to the pathogenic control strain NH48, the non-pathogenic control strain Fo47 and

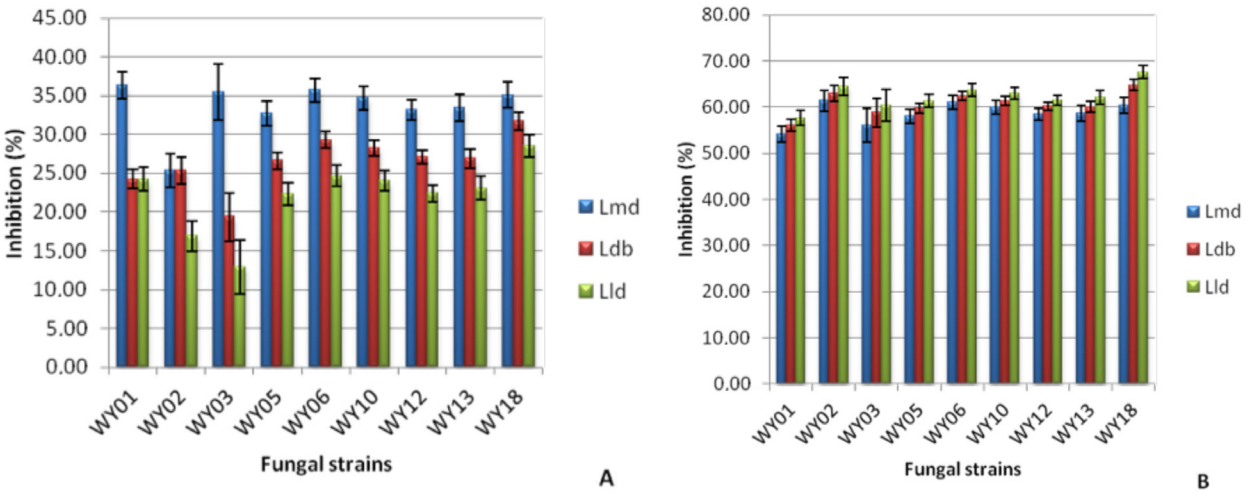

Fig. 1. Growth inhibition analysis of *Fusarium* spp. by LAB after 6-day incubation. A, on PDA medium; B, on MRSA medium.

Table 1. Growth inhibition of *Fusarium* spp. by LAB using overlay assay. +++, no fungal growth on > 8% of plate area; ++, no fungal growth on 3 to 8% of plate area; +, no fungal growth on 0.1 to 3% of plate area; –, no visible inhibition

	WY01	WY02	WY03	WY05	WY06	WY10	WY12	WY13	WY18
Control	–	–	–	–	–	–	–	–	–
Lmd	+++a	++b	+++	+++	+++	+++	+++	+++	+++
Lld	+c	++	¬¬–d	++	++	++	++	++	+++
Ldb	+	–	–	+	+	+	+	+	++

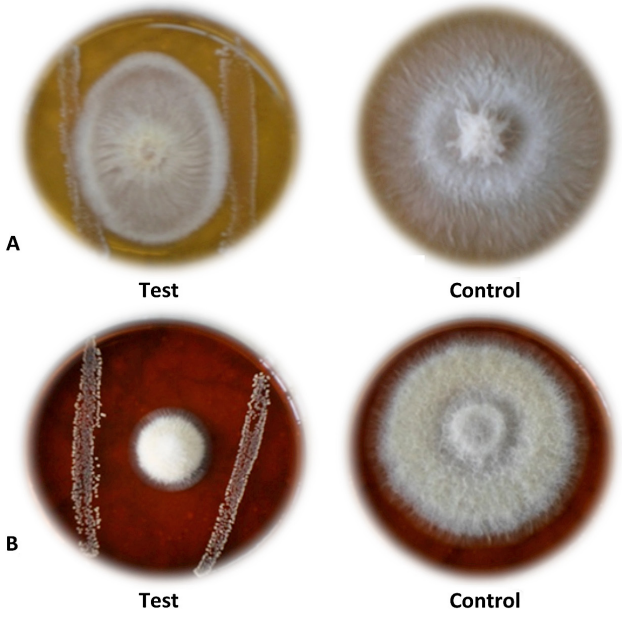

Fig. 2. Growth inhibition of WY05 by Lld after 6-day incubation. A, on PDA medium; B, on MRSA medium.

negative control prepared with MLM free fungal cells. Percentage of disease was 100% for *F. oxysporum* strains, between 46.66 and 80% for *F. redolens* strains and 36.66 and 50% for *F. solani* strains. Control plants remained asymptomatic (Table 2).

Antagonistic assays

In vivo antagonistic assays confirmed the results from *in vitro* inhibition. All LAB had significant antifungal effect against all nine fungal isolates ($P < 0.05$). Addition of LAB in soil mixture significantly reduced incidence of pathogenicity and development of plant rots. LAB activity varied significantly between 76 and 94% for Lmd, 69 and 85% for Ldb, and 59 and 78% for Lld. Thus, highest suppression efficiency was by the Lmd strain followed by Ldb and Lld. There was no significant difference of the fungal inhibition between the different fungal species ($P > 0.05$), and all these were inhibited by the three LAB. Figure 4 shows the typical symptoms of crown and root rot on infected and non-treated plants, compared with control plants, on which development of healthy roots occurred.

Discussion

In this study, *Fusarium* species were isolated from several infected plant parts of tomato cultivated in North Algeria and identified as *F. oxysporum, F. redolens* and *F. solani* by sequencing the TEF 1-α gene. According to Rezaee et al. (2018), morphological and molecular detection of fungal strains in a region is very important for disease management to achieve higher yield under specific natural conditions. All strains of *Fusarium* obtained were pathogen against tomato plants and caused crown and root rot. *Fusarium* species such as *F. oxysporum, F. solani, F. redolens, F. commune, Fusarium rolfsii* and *Fusarium incarnatum-equiseti* species complex MLST type 5 have been noted as severely virulent species of pathogenic fungi that often infect tomato plants (Hamini-Kadar et al. 2010; Si Mohammed et al. 2016; Husain et al. 2017; Yezli et al. 2019).

New alternative strategies implementing biological control by the use of lactic acid bacteria, regarding their

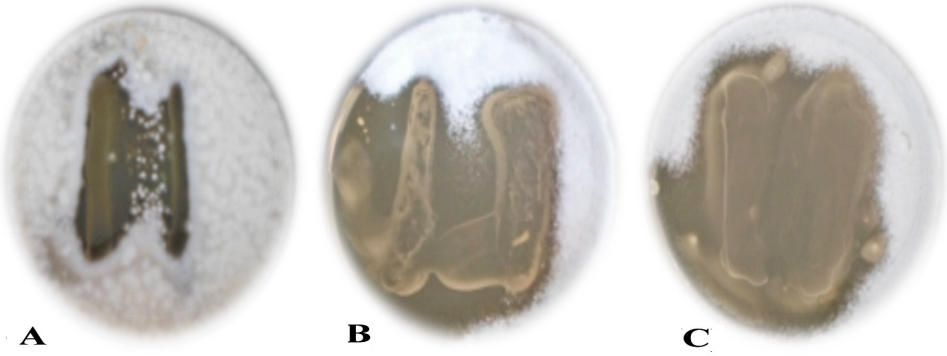

Fig.3. Growth inhibition of WY05 by LAB on PDA medium after 72-h incubation. A, Lld; B, Ldb; C, Lmd.

Table 2. Species identification and pathogenicity percentage of *Fusarium* isolates

Specimen voucher	GenBank accession No.	Species	Similarity with Genbank nucleotide database (%)	Pathogenicity (%)
WY01	MG973088	*F. oxysporum*	99.85%	100%
WY02	MG973089	*F. solani*	100%	50.00%
WY03	MG973090	*F. oxysporum*	99.85%	100%
WY05	MG973091	*F. redolens*	99.85%	76.66%
WY06	MG973092	*F. redolens*	99.85%	80.00%
WY10	MG973093	*F. solani*	100%	36.66%
WY12	MG973094	*F. solani*	99.85%	43.33%
WY13	MG973095	*F. redolens*	99.85%	46.66%
WY18	MG973096	*F. oxysporum*	100%	100%

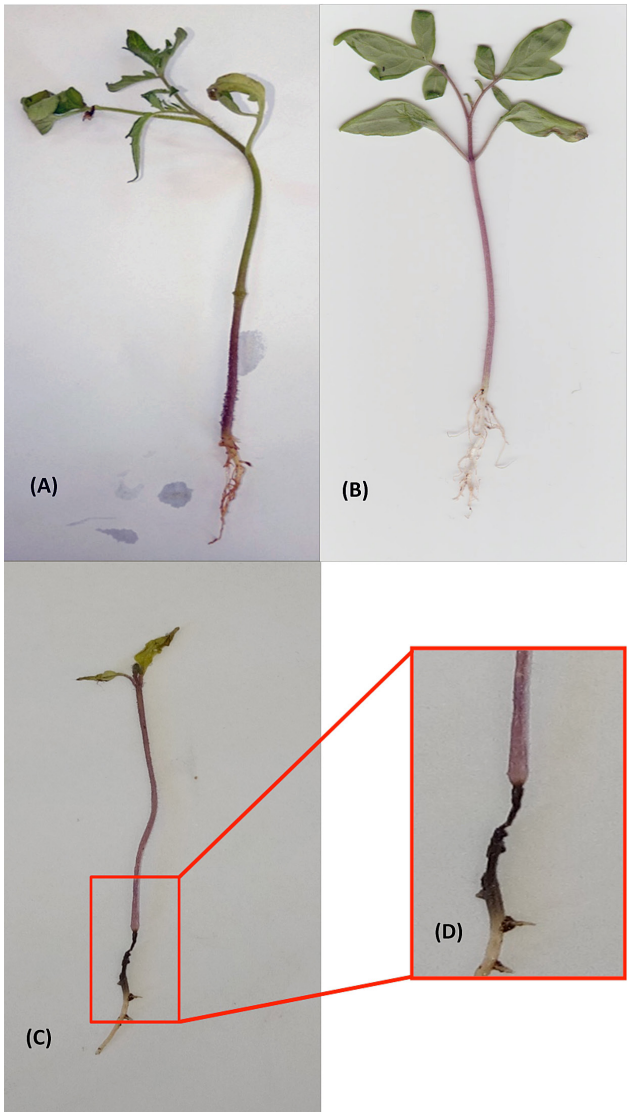

Fig.4. Pathogenic and *in vivo* antagonistic assays. A, control plant (non-treated and non-infected); B, test plant infected with WY01 isolate and treated with Lmd strain; C, test plant infected with WY01 isolate and non-treated. D, typical symptom of crown and root rot on infected and non-treated plant.

safety and practical use, were explored to understand the relation between the pathogen and the antagonistic bacteria, in order to control *Fusarium* diseases. Results of this work show that the three LAB used in this study had anti-*Fusarium* effect, which differed depending on the culture media, fungal strain and lactic strain. These results are concordant with those of Juodeikiene et al. (2018), who reported the antifungal activity and the application of LAB for reducing *Fusarium* mycotoxins in malting wheat grains. This inhibition is induced by the production of antimicrobial LAB compounds such as bacteriocins and organic acids (Svanström et al. 2013), which inhibit different fungi like species of *Fusarium* (Lavermicocca et al. 2000).

Anti-*Fusarium* activity exerted by LAB was significantly higher on MRSA medium compared with PDA medium. This study also showed that the effect of *Leuconostoc mesenteroides* subsp. *dextranicum* was significantly greater than those of *Lactococcus lactis* subsp. *diacetylactis* and *Lactobacillus delbrueckii* subsp. *bulgaricus* on PDA medium. The performance of LAB on MRS medium is related to the existence of compounds that induce gene expression of metabolite secretion in this medium and which act synergistically with LAB metabolites. However, *Lactococcus lactis* subsp. *diacetylactis* have a significantly greater effect than for the other LAB used on MRSA medium (Dalie et al. 2010; Schillinger, Villarreal 2010; Laref, Guessas 2013; Zebboudj et al. 2014). It might be explained by the growth performance of *Fusarium* species on PDA medium, which promotes mycelia growth due to addition of vegetal compounds (Desai et al. 2016).

In vivo antagonistic results confirmed that the LAB used in this study inhibits growth of different phtytopathogenic *Fusarium* species that cause tomato crown and root rot. Lmd was significantly more efficient then Ldb and Lld. Results showed also good development of plant roots in the presence of LAB. These results are in concordance with those of Hamed et al. (2011), who observed that antagonistic activity of LAB in *in vivo* conditions was higher then in *in vitro* conditions. Dhamale et al. (2015) reported that

F. oxysporum had also antagonistic activity in presence of LAB and decreased the growth of tomato seeds. This can be associated with the pathogenic variability of *F. oxysporum* (Henni et al. 1994).

This study suggests that the three new LAB isolated from camel milk can be used to significantly reduce the proliferation of different phytopathogenic *Fusarium* species. We suggest that we can use LAB cell cultures and metabolites to reduce incidence of *Fusarium* growth. LAB cells can be used *in vitro* and *in vivo* to inhibit the fungi and promote plant roots growth. Further studies should be carried out to study the potential efficacy of LAB under different environmental conditions.

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