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RESEARCH ARTICLE

Lactic Acid Bacteria: Antimicrobial activity and *in vitro*, *in vivo* studies of LAB activity on Fusarium oxysporum infected tomato seeds

Kavita Sarjerao Dhamale[1], Pragati Devidas Sonawane[2], Ashvini Suryakant Jaybhaye[3], Pavan Chand Akkiraju[4*]

1. Assistant Professor, Department of Biotechnology, P.V.P. College of Arts, Science & Commerce, Pravaranagar, Loni – 413713, Ahmednagar (Dt.), Maharashtra.
2. Department of Biotechnology, P.V.P. College of Arts, Science & Commerce, Pravaranagar, Loni – 413713, Ahmednagar (Dt.), Maharashtra.
3. Department of Biotechnology, P.V.P. College of Arts, Science & Commerce, Pravaranagar, Loni – 413713, Ahmednagar (Dt.), Maharashtra.
4. *Assistant Professor, Department of Biotechnology, P.V.P. College of Arts, Science & Commerce, Pravaranagar, Loni – 413713, Ahmednagar (Dt.), Maharashtra.

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Corresponding Author

Dr. Pavan Chand Akkiraju

Abstract

Lactic Acid Bacteria (LAB) being a group of microorganisms, are gram positive, acid-tolerant, non-sporulating *cocci* or *bacilli*. The study of lipolytic and proteolytic activities of LAB is essential to understand the biological properties of LAB. Currently, the antimicrobial activity of LAB is of major concern in plant biology. It is necessary to study the antimicrobial activity of LAB to establish a standard methodology for LAB utilization in Drug development. The current study aims to isolate and characterize LAB, to check their lipolytic, proteolytic, antimicrobial activities along with their antibiotic sensitivity. The antifungal activity of LAB on *Fusarium oxysporum* has been observed *in vitro and in vivo*. The results showed that, LAB has no lipolytic activity on Tween-20 and showed proteolytic activity. LAB showed antimicrobial activity for *P. aeruginosa*, *S. typhi*, *E. coli*, *B. subtilis* and *C. albicans*. LAB showed antibiotic sensitivity for Ampicillin, Amoxicillin, Nitrofurantion, Ciprofloaxin, Cotrimoxazole, Streptomycin, tetracycline, Pencillin, Kanamycin and resistance for gentimycin and colistin. The *in vitro* studies showed that LAB has 72.42% inhibitory property over *F. oxysporum* growth, indicating that they control the fungal action. The *in vivo* studies showed that *Fusarium* has the capacity to suppress LAB activity during seed germination.

INTRODUCTION

Lactic acid bacteria (LAB) include a large number of bacteria with lactic acid production capacity and have many industrial applications. They are Gram-Positive and non-motile organisms. They show Indole, Methyl-red and nitrate reduction tests as positive and Voges-Proskauer, Catalase and Oxidase production and Citrus-utilization tests as negative. They forms white colonies on MRS Agar plates and appear as non-spore forming *bacilli* or *cocci*, which yields lactic acid on carbohydrate fermentation. Due to their ability to withstand high acidic conditions, LAB extends their application in various products development under different conditions (Ikeda *et al.*, 2013). The LAB members of industrial importance are *Lactobacillus* and *Leuconostoc* (Food industry), *Carnobacterium* and *Pediococcus* (Bacteriocin production), *Aerococcus* and *Vagococcus* (Biopharmaceutical industry), *Weissella* and

Enterococcus (Food and Health Industry), *Tetragenococcus* (Probiotics), *Streptococcus* and *lactococcus* (Diary industry). These organisms contribute to the production of several compounds which are essential for taste, smell, color and texture of various fermented products (Rattanachaikunsopon and Phumkhachorn, 2010).

The characterization of LAB activities is of great importance to enhance their function at Industrial level. LAB produces a variety of antimicrobial compounds and effective substances such as lactic and acetic acids, probiotics, antibiotics, bacteriocins (Abbas and Mahasneh, 2014) as well as hydrogen peroxide and carbon dioxide (Hamed *et al.*, 2013). Strains that are used as probiotics for man have been isolated from the human gastrointestinal tract and usually belong to species of the genera *Lactobacillus* and *Bifidobacterium*. However, strains belonging to species of other LAB have been used in the past as probiotics as well, such as *E. faecium, S. thermophilus, Le. mesenteroides*, *L. lactis* subsp. *lactis*, *E. faecalis*, and *P. acidilactici* (Ananou *et al.*, 2007). LAB plays an important role as preservatives in milk and milk products (Mutlag *et al.*, 2013). They are lipolytic (Guessas *et al.*, 2012) and proteolytic (Guessas *et al.*, 2007, 2012) against some microorganisms and also shows antibacterial (Guessas *et al.*, 2012, Rattanachaikunsopon and Phumkhachorn, 2010) and antifungal activity.

Major plant pathogenic fungi, which influences the economy of agriculture worldwide includes *Magnaporthe*, *Botrytis*, *Puccinia*, *Fusarium*, *Blumeria*, *Mycosphaerella*, *Colletotrichum*, *Ustilago* and *Melampsora*. Among these fungi, *Fusarium* contains more than hundred species (Abed, 2013) with notable impact over the growth of various plants. *F. graminearum* and *F. oxysporum* are the important fungi, affects food crops worldwide. Mainly, *F. oxysporum* is responsible for wilt and cortical rot diseases in most of the economically important plants (Hasan *et al.*, 2013). Its habitat includes arctic, desert, tropical soils mainly and also involves cultivated soils. It is dispersed all over the world and the specific fungus is hosted by potato, tomato, sugarcane, garden bean, cowpea, watermelon, cucumber, Muskmelon and prickly pear.

The current work was focused on, a) Isolation & Identification of LAB, b) Lipolytic Activity and c) Proteolytic activity of Isolated Microorganisms, d) Screening for Antimicrobial Activity, e) test for antibiotic sensitivity, f) *In vitro* screening of LAB activity against *F. oxysporum* and g) *In vivo* screening of LAB activity against *F. oxysporum*.

Materials and Methods

Sample collection:

Two samples of traditionally available buttermilk were collected from the local market of Loni, Maharashtra. One sample was obtained from the local rice, through natural farming (David M, 2013). The second sample was collected from the curd directly. Both the samples were transported to the Department of Biotechnology, PVP College, Loni, under safe conditions, where further studies were performed. For the current study, the second sample obtained from the curd was used.

Isolation and Identification of Lactic Acid Bacteria

Ten grams of butter was homogenized with 90 ml of sterile NaCl (0.7%) solution and then, a tenfold serial dilution was carried out. The lactic acid bacteria were isolated on MRS (pH 6.5 ± 0.02) medium and incubated at 37^{o}C for 24hrs. The lactic acid bacteria were maintained freshly throughout the experimental work.

Fungus maintenance

The fungus sample of *F. oxysporum* was generously given by Avishkar Biopharm, Pravaranagar, Loni. The fungal strains were maintained properly through regular sub-culturing on Potato Dextrose Agar (PDA). The fresh cultures were used throughout the experiments.

Lipolytic activity of LAB

The lipolytic activity of lactic acid bacteria was tested by using the method described by Guessas *et al*. (2012). A 20 ml medium containing LAB was allowed for incubation and examined frequently for the lipolytic activity over Tween-20.

Proteolytic activity of LAB

The Plate count agar method was used to understand the proteolytic activity of LAB. In this procedure, 1% and 2% skim milk were used as the protein substance. The clear zone resulted around the well was considered as the positive and absence is recorded as the negative.

Screening for Antimicrobial activity

The lactic acid bacteria were tested for antifungal activity. Some samples of LAB showed antifungal activity, especially *F. oxysporum*, *Candida albicans* and *Bacillus subtilis*. LAB sample was poured in to the wells of the nutrient medium prepared and observed for the zone of inhibition over the fungal samples.

Test for antibiotic sensitivity of LAB

The LAB isolates were grown in MRS broth and incubated for 24 hours. 5 ml of LAB was mixed in 100 ml of Mueller-Hinton Agar and poured in to sterile Petri plates. The plates were left to solidify and then, different antibiotic discs were placed on agar and pressed gently. The plates were incubated at 37^{o}C for 24 hours and observed for resistance. The absence of a zone of inhibition was confirmed as resistance and the presence of zone was considered as positive.

In vitro* screening of LAB activity against *Fusarium oxysporum

Erlenmeyer flasks of 250 ml containing Potato Dextrose broth were taken for the *in vitro* assays of antifungal activity by LAB. Separate flasks for *F. oxysporum* alone and *F.oxysporum* with LAB were taken and incubated at 27 ± 1^{o}C on orbital shaker. Following 7 days of incubation, the resulted fungus was filtered, washed well and dried at 50-55^{o}C. The growth inhibition (GI) Percentage was calculated as follows:

$$GI\ (\%) = Co - Cf / Co \times 100\%,$$

Where, Co is the dry weight of fungal mycelium (control), Cf is the dry weight of fungal mycelium after inhibition by LAB.

In vivo* screening of LAB against *Fusarium oxysporum

Wet filter papers were mounted into Petri plates and 10 tomato seeds were placed on top of the filter paper. Before leaving the plates for incubation, the seed were treated as follows: 1) only with LAB, 2) Only with *F.oxysporum*, 3) Both LAB and *F.oxysporum* and 4) no treatment, treated as control. The seeds were incubated at 37^{o}C for 10 days. The average values of 10 seeds were used in the analysis.

Results

1. Lipolytic activity of LAB

After continuous evaluation for lipolytic activity on Tween-20 by LAB, the results were found to be negative. The current strain used in the study was unable to digest the lipid portion.

2. Proteolytic activity of LAB

Proteolytic activity was resulted slightly in 1% of skimmed milk in PCA and there was no activity observed with 2% skimmed milk (Fig. 1)

3. Antimicrobial activity of LAB

LAB showed antimicrobial activity against different bacteria and fungi by forming zone of inhibition (Table I, Fig. 2 - a, b, c, d, e and f). These results indicate that LAB strains are capable of producing certain inhibitory substances, which acts on the pathogenic bacteria.

4. Test for antibiotic sensitivity of LAB

LAB showed antibiotic sensitivity for, Amoxicillin (AMC) (30 µg), Nitrofurantion (NIT, FT) (300 µg), Ciprofloaxin (CIP) (10 µg), Cotrimoxazol (COT) (25 µg), Streptomycin (S) (10 µg), Ofloxacin (OFX) (10 µg), Tetracycline (TE) (30 µg), and resistant for Ampicillin (AMP) (10 µg), Gentimycin (GEN) (10 µg) and Colistin(CL) (10 µg) (Table II, Fig. 3 – a, b, c, d and e)**.**

5. *In vitro* screening of LAB against *Fusarium oxysporum*

The dry weight of control (Fungus only) was measured as 0.367gm and the same of bacteria and fungus mixture resulted as 0.101gm. The percentage of growth inhibition was calculated as Co – Cf / Co x 100% => 0.367-0.101/Co× 100% => 0.266/0.367 × 100 i.e. 72.42. LAB showed about 72.42% inhibition in *F. oxysporum* growth, indicating their ability to control the fungal action.

In vivo* screening of LAB against *Fusarium oxysporum

In vivo screening showed that LAB and control showed similar growth at shoot (5.2 and 5.7, respectively), where as they differed drastically at root level (3.8 and 10.3, respectively). From this result, it is clear that LAB can induce growth at shoot level, but cannot induce the root growth. *F.oxysporum* showed negative relation with the growth of the seeds and resulted in 2.8cm and 1cm for shoot and root lengths respectively. This confirms that, *F.oxysporum* is a threat to the seed's growth. When both LAB and *F.oxysporum* applied on the seeds, there was no growth observed in any of the seeds (Table III, Fig.4).

Fig.1

LAB with Proteolytic activity (1% skimmed milk)

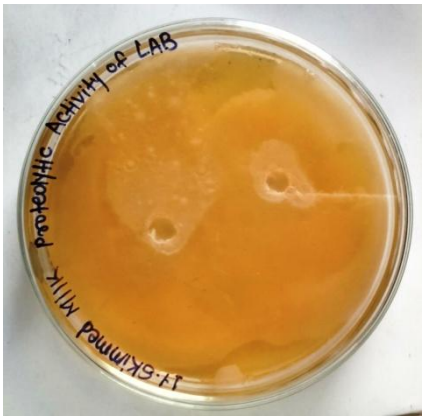
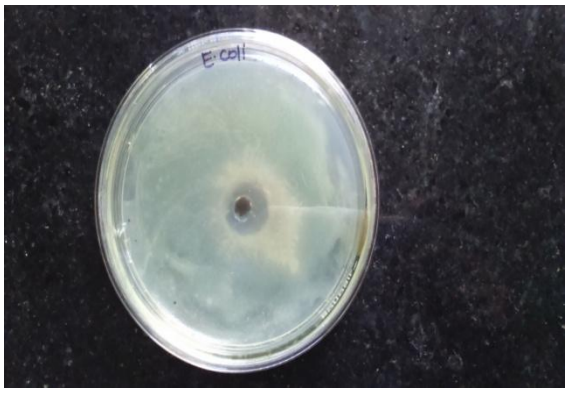
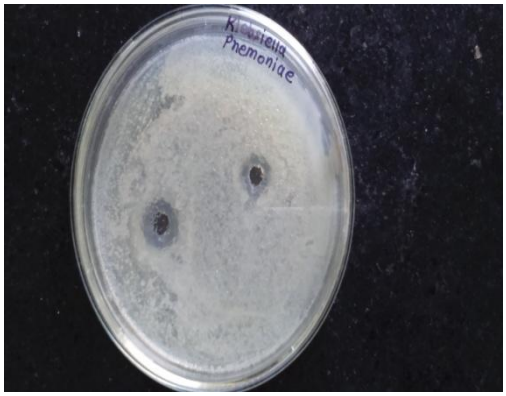


Fig.2

Antimicrobial activity of LAB

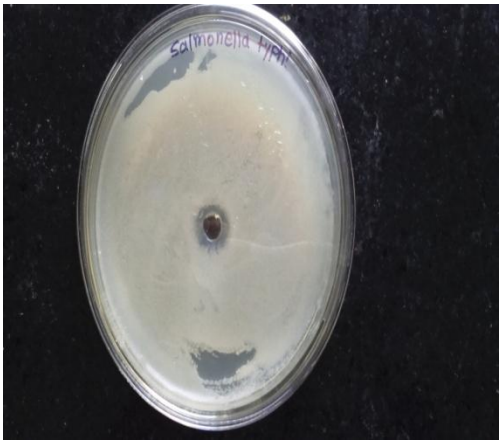


a) LAB activity on *E. coli*

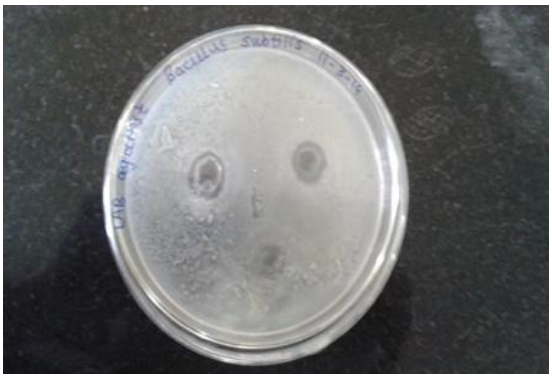


b) LAB activity on *Klebsiella pneumoniae*

c) LAB activity on *Psudomonas aeruginosa*



d) LAB activity on *Salmonella typhi*



e) LAB activity on *Bacillus subtilis*

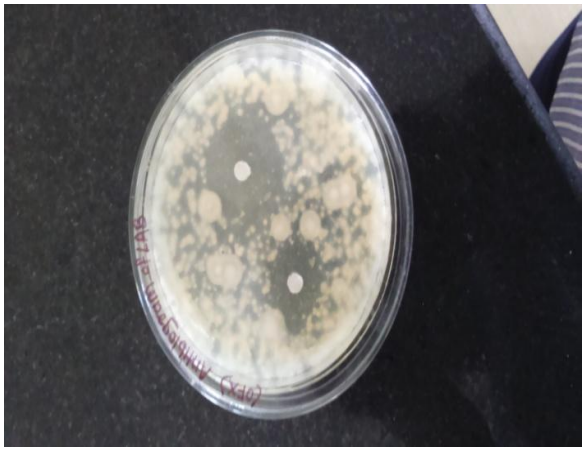
f) LAB activity on *Candida albicans*

Fig.3

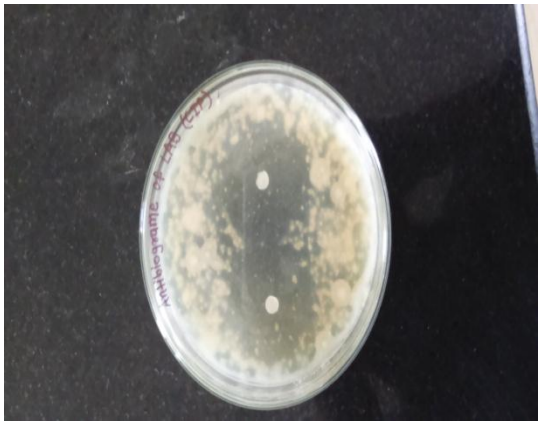
Antibiotic sensitivity of LAB

a) Antibiotics activity on LAB: S, TE, AMP, CL, CIP, COT, GEN, NIT

b) Activity of FT on LAB



C) Activity of OFX on LAB



d) Activity of CIP on LAB

e) Activity of AMC on LAB

Fig.4
Tomato seeds treated with LAB and *F.oxysporum* and their comparison with control

Table I

Antimicrobial activity of LAB on bacteria and fungi and their zone of inhibition (mm)

Name of Microorganism	Zone of inhibition (mm)
Salmonella typhi	1
Escherichia coli	1.5
Bacillus subtils	1
Psudomonas aeruginosa	1.1
Candida albicans	0.7
Klebsiella pneumonia	0.7

Table II

Antibiotic sensitivity on LAB and their zone of inhibition (mm)

Antibiotic	Zone of inhibition (mm)
Amoxicillin (AMC)	2.7
Nitrofurantion (NIT,FT)	1.5
Ciprofloaxin(CIP)	2.0
Cotrimoxazol (COT)	0.7
Streptomycin (S)	1.0
Tetracycline (TE)	1.3
Ofloxacin (OFX)	1.7

Table III

Average shoots and root lengths of tomato seeds that are exposed to LAB and *F.oxysporum* separately and in combination

	Control	LAB Only	*F.oxysporum* only	LAB+ *F.oxysporum*
Shoot length (cm)	5.7	5.2	2.8	0
Root length (cm)	10.3	3.8	1	0

Discussion

The lactic acid bacteria are predominant in total micro flora of human system. The continuous studies over LAB have already proven that LAB is the dominant microorganisms available in all traditional dairy products. The current study has focused on various aspects of LAB and mainly antibiotic activity over different microorganisms, especially *F.oxysporum.*

In the current study, LAB was observed for its lipolytic and proteolytic abilities. LAB has not showed any lipolytic activity on Tween-20, but showed proteolytic activity on skimmed milk at 1%. Guessas *et al*. (2012) had a total of 76 isolates of LAB, out of which only two isolates (*Lactobacillus delbrueckii* subsps. *delbrueckii* and *Lactobacillus delbrueckii* subsp.*bulgaricus*) found to be lypolytic in their action. By this information, it is clear that LAB has less ability to degrade the lipids. This particular property can be used in preparation of antibiotics and milk products by without disturbing the natural LAB environment.

LAB has showed proteolytic activity in 1% skimmed milk, but not in 2% of it. Similar results were given by Guessas *et al*. (2007, 2012) and indicted that *Lactococcus lactis* subsp. *cremoris* showed slight reaction in 1% and has no activity in 2%. The same authors also showed that *Lactobacillus plantarum* has no proteolytic activity. Donkor *et al*. (2007) also confirmed that LAB has the proteolytic activity.

The antimicrobial activity of LAB is of major importance and discussed by many authors. Savadogo *et al*. (2004) showed that, LAB has the ability to synthesize bacteriocin, which led to the formation of zone of inhibition against various pathogenic strains. The major species included were *Enterococcus faecalis*, *Bacillus cereus* and *Staphylococcus aureus*. The most inhibited strains were of Gram positive nature and only one Gram negative strain (*Escherichia coli*) was found. In the current study, the LAB showed antimicrobial activity against *Salmonella typhi*, *Escherichia coli*, *Bacillus subtils*, *Psudomonas aeruginosa*, *Candida albicans and Klebsiella pneumonia.*

Several authors (Vaughan *et al*., 1994; Guessas *et al.,* 2007) showed antimicrobial activities of LAB against various pathogens. But, the proportion of total strains tested to the strains showing antimicrobial activities is found to be minute. Askouul and Saiah (2014) found that only one out of 93 strains showed antimicrobial activity. Kazemipoor *et al.* (2012) were isolated only one strain with antimicrobial activity from 221 isolates. Vaughan *et al*. (1994) showed the ability of LAB against *Staphylococcus aureus*. Pundir *et al.* (2013) showed that LAB (two different species - *Lb.brevis* and *Lc. Lactis)* has inhibitory action over *E. faecalis* (9mm), *Streptococcus xylosus* (16mm and 10mm), *Bacillus linens* SR3 (15mm) and *Staphylococcus aureus* (4mm). (Rattanachaikunsopon and Phumkhachorn, 2010) presented various bacteriocins from LAB which results in antimicrobial activity. The antibiotic sensitivity of LAB observed in the current study showed similar results with Mutlag *et al*. (2013). The same authors reported that LAB is not showing any sensitivity towards Colistin, which is similar to the current results. But, the current study is different with the above authors in LAB response to gentamicin and ampicillin. In the current study, we confirm that LAB is resistant to gentamicin activity.

In *in vitro* efficacy, the current study resulted with 72.42% inhibition to *F. oxysporum* growth, which can be compared with the results of Hamed *et al.* (2011), where high inhibition effect of LAB against S. *rolfsi* was observed by *LB-4* strain. *In vitro* studies of Murthy (2012) showed that LAB can act as Plant Growth Promoting bacteria. The current study confirms this activity of LAB.

In *in vivo conditions,* Hamed *et al.* (2011) showed that LB-1 and LB-5 showed higher antifungal activity under *in vivo* tests. The current study also indicates similar results with the presence of LAB. But, the current results showed that, *F.oxysporum* is showing antagonistic activity with LAB and resulted in ceasing of seed growth when mixed with LAB. This indicates that LAB was interfered by *F.oxysporum*, which intern resulted in the successful growth of the seed.

Conclusion

From past few decades, number of studies has been conducted over the antibacterial activities of Lactic acid bacteria. From these studies, it is possible to isolate and characterize LAB and the consecutive studies were made easily available. The production of bacteriocins made LAB as antibacterial or antimicrobial and is also recognized as Plant Growth Promoting bacteria. as *F.oxysporum* is becoming resistant and is capable of interfering with the activity of LAB, the antimicrobial studies of LAB are of great importance in the nearby future. The future aspects of the related research area should include *in vitro* and *in vivo* tests of LAB and *F.oxysporum* activities on the seed growth to understand the complete mechanisms involved in the seed growth against the fungal pathogen.

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