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Investigation of the Antifungal Activity of *Lactobacillus* against *Aspergillus Niger* and *Penicillium Oxalicum*

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Abstract

The study investigated the antifungal activity against Aspergillus niger CBS 76997 *and Penicillium oxalicum* 20B *of several Lactobacillus strains (LAB). The in vitro antifungal activity of cell-free supernatants and biomass of Lactobacillus strains against the growth of A. niger* CBS 76997 *and P. oxalicum* 20B *was determined by monitoring the fungal colony diameter over time by spot inoculation method and double layer method. The antifungal effect was dependent on LAB and fungi strains. The control sample of A. niger and P. oxalicum reached the mycelial growth diameter 100% (85 mm) after 5 days and 10 days, respectively. The cell-free supernatant and biomass of LAB could inhibit the growth of tested fungi ranging from 35.3% to 84.7% and from 30.6 to 100%, respectively. When inoculation, LAB can delay the spore-forming process from 2 to 10 days. The research results demonstrated the potential application of lactic acid bacteria as a biological inhibitor of fungal growth in the preservation and processing of food products.*

Keywords: *Aspergillus Niger*, antifungal activity, lactic acid bacteria, *Penicillium oxalicum.*

1. Introduction

Fungi are generally found in soil, air, and plants and can contaminate a variety of foods and animal feeds. *Aspergillus* and *Penicillium* are very important spoilage in food. It is known commonly to cause black mold in fruits and vegetables like grapes, apricots, onions, and peanuts, leading to food contamination or spoilage. According to a report by the Food and Agriculture Organization of the United Nations (FAO), hundreds of billions of dollars are lost across the world each year due to fungal and toxin infestation of crops, resulting in loss of food value and consequent significant economic losses [1]. Around 931 tons/year of food was estimated to be wasted globally, with fungi spoilage being the main reason. In addition, fungi are capable of producing a mycotoxin (aflatoxins, ochratoxins, trichothecenes, zearalenone, fumonisins, and patulin), considered a public health concern which is associated with both toxicity and carcinogenicity.

Despite enormous investments in crop production, technological preservation in the harvest and post-harvest stages remains lacking. Some physical and chemical preservatives have been applied to control fungi growth. Washing and drying are commonly used in cereal storage, but they are low in efficiency since they can only remove a small percentage of mould and mycotoxins. As a control tool for fungal growth, food industries react by adopting good manufacturing practices and generally using chemical preservatives such as natamycin, sulfite, potassium sorbate or sodium benzoate. However, major drawbacks of chemical preservation methods are the requirements of precise pressure and temperature controls and the chemical residues in post-treatment food products, making them technically difficult and costly to implement in practice [2].

Among many alternative bioactive natural antifungal compounds proposed to date, lactic acid bacteria have become a new trend of interest recently. Lactic acid bacteria (LAB), recognized as a safe and qualified additive by the Food and Drug Administration (FDA), have been shown to inhibit fungal growth and degrade mycotoxins [3]. LAB could inhibit fungal spore germination and mycelial growth by competing for growth space and by secreting nutrient-rich microbial active substances. Antifungal LAB produces active metabolites such as lactic acid, acetic acid, cyclic dipeptides, phenylacetic acid, hydroxy fatty acids, and 3-hydroxy propionaldehyde, which have been shown to be associated with the antifungal effect of LAB [3]. Some LAB strains have demonstrated antimicrobial activity from producing antimicrobial compounds that can be used as natural preservatives in a broad range of food products. However, to the best of our knowledge, the research on the antifungal effects of LAB in Vietnam was limited.

Hence, the objective of this study was to investigate fungal inhibition of some LAB. The aim is

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to provide an excellent primary raw material for biological preservatives used in feed and food production.

2. Materials and Methods

2.1. Materials

This study used 05 LAB strains, including *Lactobacillus acidophilus* TM, *Lactobacillus acidophilus* VAST, *Lactobacillus fermentum* HA7, *Lactobacillus rhamnosus* GG, and *Lactobacillus paracasei* B21060. These strains were collected from the laboratory of the Department of Food Technology, Hanoi University of Science and Technology.

Two fungi, *Aspergillus niger* CBS 76997 and *Penicillium oxalicum* 20B, were kindly provided by Food Industries Research Institute (Fig. 1. and Fig. 2).

The LAB strains and fungi spore were kept in 20% glycerol solution and stored at -80 ºC.

Fig 1. *Aspergillus niger* CBS 76997

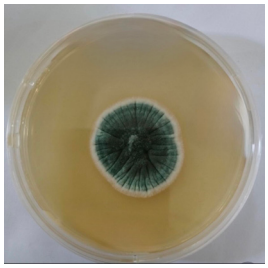

Fig 2. *Penicillium oxalicum* 20B

2.2. Methods

2.2.1. Preparation of LAB culture and cell-free supernatant

The LAB were activated in MRS broth (HiMedia, India) at 30 ºC for 48 hours in anaerobic condition, then streaked on MRS agar (HiMedia, India). After 48 hours, when the colonies grew, the agar plate was kept at 4 °C for a short time. The LAB strains were inoculated into MRS broth and incubated for 24 hours to 48 hours at 30 °C.

After incubation, the cell culture was determined for pH, total acid content and optical density at wavelength 600nm at two-time points, 24 hours and 48 hours.

Cell-free supernatant (CFS) was prepared by centrifuging the broth in a centrifuge at 6000×*g* for 10 min at 6 °C (Hermle, Germany). The LAB supernatant was filtrated using a sterile filter (0.2 µm-pore-size filter, Millipore).

2.2.2. pH determination method

After incubation, the bacterial culture medium is brought to room temperature (~25 °C). A pH meter is used to measure the pH value of the bacterial culture medium.

2.2.3. Total acid content determination method

The total organic acid content was determined using the titration method with an alkali solution, as introduced by Le Thanh Mai (2009) [4].

The total acid content is calculated as formula:

$$\frac{A \times 0{,}009 \times 100}{V} \text{ (g/100mL)} \quad (1)$$

A - Volume of used NaOH 0,1M (ml)

V - Volume of sample solution (V = 5 ml)

0,009 - Conversion factor (weight of lactic acid (g)/1 ml NaOH 0,1M)

2.2.4. Optical density

The inoculum's optical density (OD) was measured at 600 nm. The blank sample was MRS broth.

2.2.5. Determination of antifungal activity of cell-free supernatant of LAB by spot inoculation method

The antifungal assay was carried out on *A. niger* CBS 76997 and *P. oxalicum* 20B. CFS of LAB was prepared as described in the 2.2.1 section. 10% of CFS was added to Petri plates, and then 90% Yeast Pepton Dextrose (YPD, HiMedia) agar medium was added at 45 ºC, stirred, and waited until it solidified. 5-day-old fungi spores were inoculated in the centre of these plates using the spot inoculation method (Fig. 3). The plates were then incubated at 30 °C for 5 days for *A. niger* and 10 days for *P. oxalicum*. The mycelia were observed, the diameter of the mycelia was measured daily, and the spore-forming time was recorded [5]. This assay was performed in triplicate.

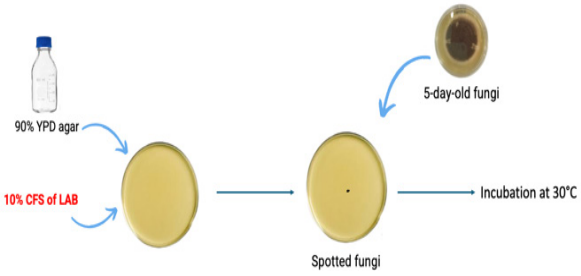

Fig. 3. Evaluation of the antifungal activity of CFS of LAB

YPD agar plates added 10% sterilized distilled water and inoculated with only fungi were used as control positive and YPD agar added CFS of LAB plates were used as control negative.

The inhibition rate (*IR*) was calculated as follows [6]:

$$Inhibition\ rate\ (\%) = \frac{\text{Dcontrol} - \text{Dtest}}{\text{Dcontrol}} \quad (2)$$

where:

$D_{control}$ is the diameter of the mould colony on the control positive sample plate (mm);

D_{test} is the diameter of the mould colony on the test sample plate (mm).

2.2.6. Determination of antifungal activity of LAB biomass by double layer method

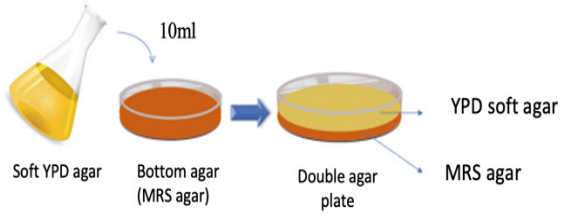

Fig. 4. Preparation of double-layer agar plate

The antifungal ability of the cell biomass was evaluated using the double-layer plate method [7]. After activation of the LAB, the cell culture was measured by the OD and adjusted to the concentration of 10^6 CFU/mL. 100 µL of LAB cell culture was grown in MRS agar medium using the spread plate method and incubated for 24 hours at 30 °C as described in 2.2.1 section. After growing, soft YPD (0,7% agar) was poured on to the MRS layer, wait until the bilayer plate was thoroughly solidified, then spotted 5-day-old fungi spores in the centre of these plates (Fig. 4). The plates were then incubated at 30 °C for five days for *A. niger* and ten days for *P. oxalicum*. The antifungal effect of LAB strains was evaluated according to the diameter of mycelia and spore-forming time. The control sample was performed under the same condition without inoculation LAB strains. This assay was performed in triplicate.

3. Results and Discussion

3.1. Characteristics of Tested LAB

After incubation, the LAB cell cultures were determined by pH, total acid content, and OD. The results were presented in Table 1. There is no significant difference at the two culture times of 24 hours and 48 hours. The pH of the culture of the 5 strains ranged from 3.89 to 4.06, corresponding to the total acid content and OD, which also had no significant difference. Therefore, the study chose a 24-hour time point to evaluate the antifungal activity of LAB.

3.2. Antifungal Activity of LAB Cell-Free Supernatant

In this study, the antifungal assay was carried out on *Aspergillus niger* CBS 76997 and *Penicillium oxalicum* 20B. The antifungal activities of CFS of LAB were presented in Fig. 5, Fig. 6, Fig. 7 and Fig. 8.

During the incubation period, *A. niger* was initially white and changed to black after three days producing conidial spore. The edges of the colonies appear pale yellow, producing radial fissures. The mycelial diameter of *A. niger* reached 85mm after five days of incubation. For *P. oxalicum*, the mycelial diameter of this strain reached 85mm after 10 days of incubation, longer than *A. niger*.

Table 1. pH, total acid content and optical density of tested lactic acid strains

Parameters	Time	*L. fermentum* HA7	*L. acidophilus* VAST	*L. acidophilus* TM	*L. rhamnosus* GG	*L. paracasei* B21060
pH	24h	4.03 ± 0.07	4.02 ± 0.05	4.06 ± 0.02	4.03 ± 0.03	3.98 ± 0.05
	48h	4.01 ± 0.04	4.00 ± 0.04	4.06 ± 0.00	4.03 ± 0.02	3.89 ± 0.07
Total acid content (g/100mL)	24h	2.11	2.11	1.21	1.51	2.16
	48h	2.07	2.06	1.98	2.04	2.07
OD	24h	2.34	2.38	2.18	2.26	2.30
	48h	2.35	2.37	2.37	2.36	2.37

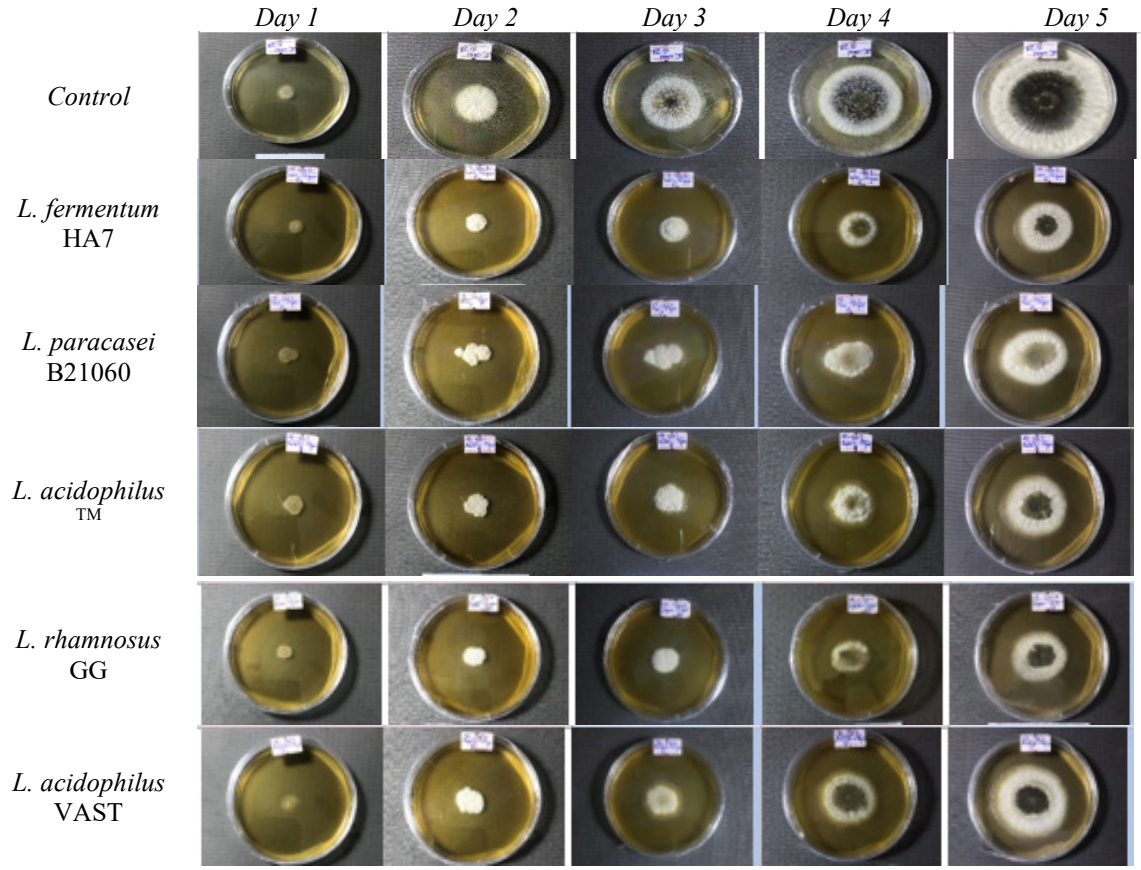

Fig. 5. Effect of cell-free supernatant of lactic acid bacteria on the growth of *A. niger* CBS 76997 within five days on YPD agar plates

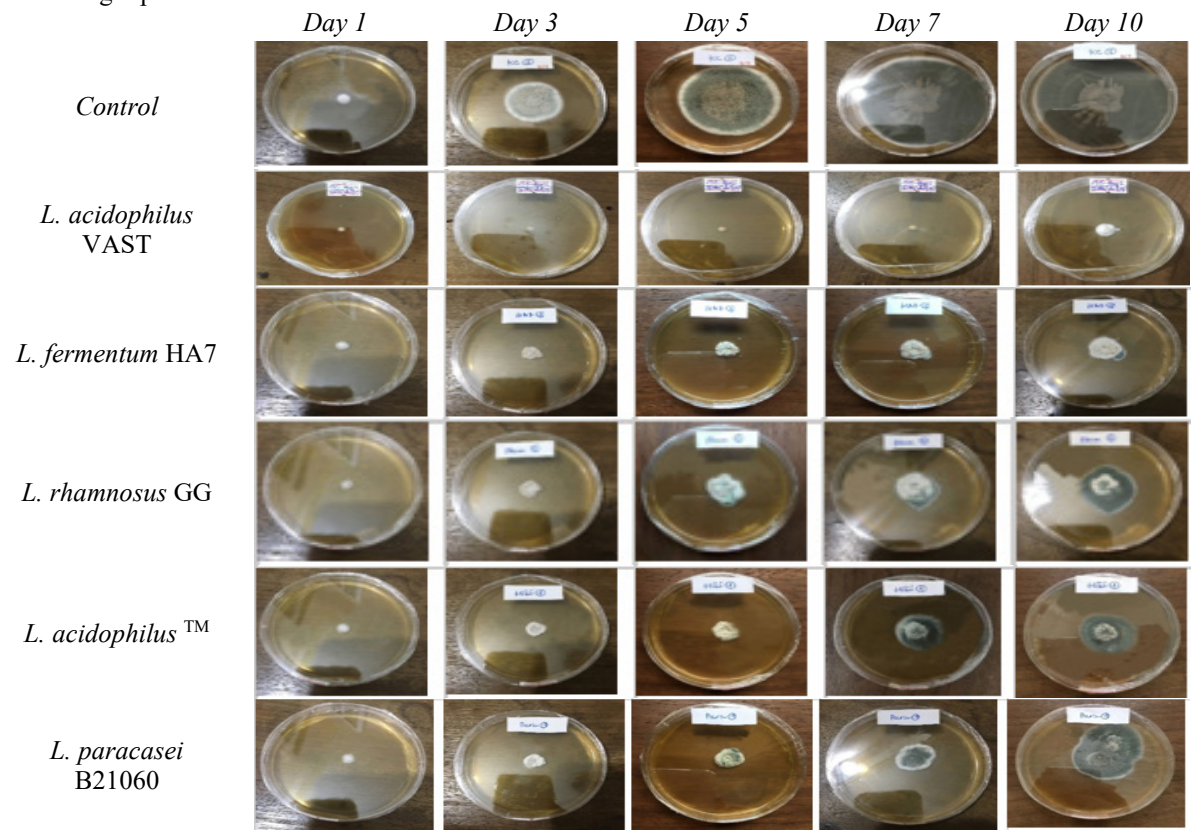

Fig. 6. Effect of cell-free supernatant of lactic acid bacteria on the growth of *P. oxalicum* 20B within 10 days on YPD agar plates

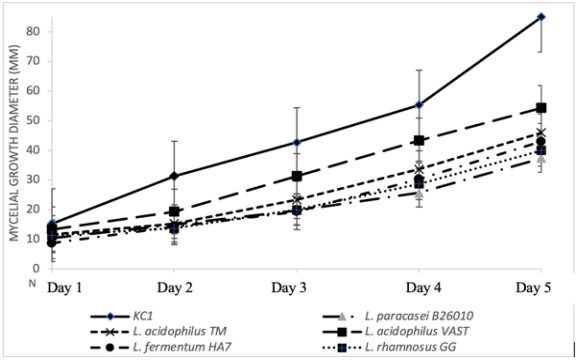

Fig. 7: Mycelial growth diameter of *A. niger* CBS 76997 within five days of co-incubation with LAB cell-free supernatant

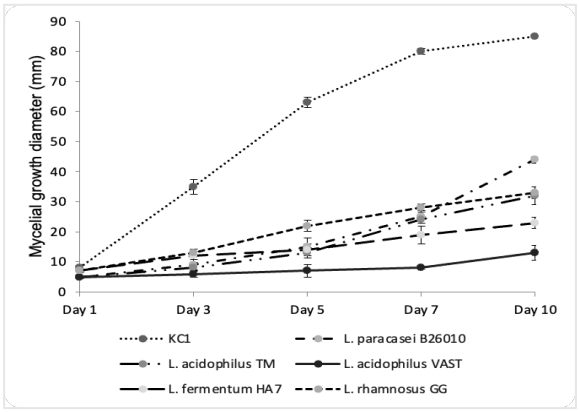

Fig. 8: Mycelial growth diameter of *P. oxalicum* 20B within ten days of co-incubation with LAB cell-free supernatant

When treated with CFS or cell biomass of LAB, the diameters of these colonies increased from slightly to significantly every day, varying among inoculated LAB species. Nevertheless, the diameters of mould colonies in all agar plates inoculated with bacteria cell biomass were visually smaller, by varying degrees, than those of the blank control. The samples supplemented with the CFS of LAB exhibited comparable mycelial growth diameters after 5 days of cultivation, ranging from 34.0 to 54.0 mm. Regarding the spore-forming process, all five strains maintained the absence of fungal spores until the 3rd day (observed by colour change). Comparing the black colour of spore formed on the mycelial by the naked eye, the samples supplemented with the CFS of *L. paracasei* B21060 demonstrated better antifungal capabilities by exhibiting a lower density of developed fungal spores (Fig. 5). This suggests that all five strains of LAB used in the experiment exhibited antifungal properties against the *A. niger* CBS 76997 fungal strain.

The CFS of five strains of LAB used in the experiment also clearly demonstrated resistance against the fungus *P. oxalicum* 20B, even exhibiting better resistance compared to the *A. niger* CBS 76997. By the 10th day of cultivation, when the control dish was fully colonized, the experimental dishes still maintained significantly smaller mycelial diameter (Fig. 6).

After ten days of cultivation, when the fungi on the control dish had fully colonized, the fungal diameter reached 85 mm, while the mycelial growth diameters in the experimental samples ranged from 12 to 45 mm. Specifically, the sample supplemented with *L. acidophilus* VAST CFS exhibited the smallest diameter, only 12.67 ± 0.58 mm. Following this, in ascending order, were *L. fermentum* HA7 (24.67 ± 1.53 mm), *L. acidophilus* TM (30.67 ± 2.31 mm), *L. rhamnosus* GG (33.67 ± 1.15 mm), and *L. paracasei* B26010 (45.00 ± 1.73 mm). Regarding the ability to inhibit spore formation, the results were similar to those for inhibiting fungal growth. Overall, all treated samples with CFS of tested LAB remained spore-free until the 5th day. *L. acidophilus* VAST could delay the spore formation until the 10th day.

Therefore, in terms of the ability to inhibit fungal growth, the *L. acidophilus* VAST CFS exhibited the best antifungal activity against *P. oxalicum* compared to the other four LAB strains.

These findings are consistent with previous studies. H. Abouloifa, *et al.* [8] evaluated the antifungal activity of CFS from *Lactobacillus* probiotic strains isolated from fermented green olives against various mold strains. The results demonstrated that the CFS from all *Lactobacillus* strains exhibited antifungal activity against the studied fungi strains, with inhibition zone diameters ranging from 12.95 to 14.4 mm and 16.1 to 17.3 mm for *A. niger* and *P. digitatum*, respectively. The growth of *Fusarium culmorum*, *Aspergillus niger*, and *Penicillium expansum* spores was inhibited by reuterin produced by *Limosilactobacillus reuteri*n R29; the CFS with the highest concentration of reuterin completely prevented the growth of all three fungal spores [9]. Each LAB strain showed varying antifungal abilities against different mould strains. This could be explained by the different compounds produced during the growth of each microorganism [9]. Additionally, each fungus exhibited varying sensitivities to the components present in the CFS.

3.3. Antifungal Activity of LAB Biomass

The biomass of 5 strains of LAB was evenly spread on MRS agar at a concentration of 10^6 CFU/mL. Subsequently, fungi spores were supplemented at 24 h after bacterial inoculation. The variation in the mycelial growth diameter was monitored to assess the antifungal capability of the

bacterial biomass. Regarding the mycelial growth diameter of the experimental samples, there was a significant decrease in the fungal inhibition zone diameter compared to the control sample. All samples exhibited good antifungal abilities, surpassing those of the sample supplemented with the CFS of the bacteria (Fig. 9).

The four samples supplemented with L. *fermentum* HA7, *L. rhamnosus* GG, *L. acidophilus* VAST, and *L. paracasei* B21060 showed the same antifungal activity with the mycelial growth ranging from 39.7 to 47.7 mm while *L. acidophilus* TM exhibited a weak antifungal activity ($D = 60 \pm 2.65$ mm) (Fig. 9). In addition, *L. paracasei* B21060 could delay spore forming two days compared to the control sample. For the remaining LAB, the antifungal activity significantly diminished on the 5th day as the mycelium grew rapidly and robustly, becoming denser compared to the 4th day.

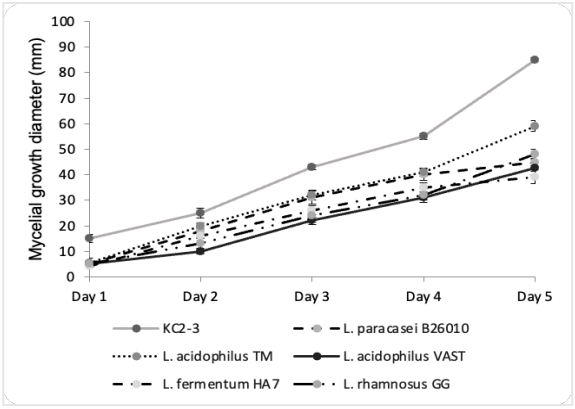

Fig. 9: Mycelial growth diameter of *A. niger* CBS 76997 within five days of co-incubation with 10^6 CFU/mL of LAB cells

For *P. oxalicum* 20B, after 10 days of cultivation, the experimental samples of all 5 LAB strains showed no signs of fungi growth, exhibiting complete inhibition against these fungi. Therefore, the presence of lactic acid bacteria combined with their metabolic byproducts after 24 hours of growth strongly influences the growth of moulds.

This finding aligns with the study of Mille-Lindblom *et al.* (2006), where they observed that when bacteria were cultured in the growth medium before mould inoculation, the mould did not grow at all. However, when the mould was inoculated concurrently with additional nutrient supplementation, mould growth proceeded and achieved biomass similar to that in the co-culture medium with mould and bacteria [10]. This suggests that *P. oxalicum* 20B is more sensitive to LAB strains biomass compared to *A. niger* CBS 76997.

Fernandez *et al.* (2017) found that the most effective LAB strains (based on the diameter of the inhibition zone) against *Penicillium chrysogenum, Mucor racemosus, Aspergillus versicolor, and Cladosporium* belonged to the *Lactobacillus* genus, especially species like *L. fermentum, L. helveticus, L. paracasei, L. pentosus, L. plantarum,* and *L. rhamnosus* [11]. In our study, the presence of LAB biomass exhibited strong antifungal properties against two tested fungi, outperforming the 10% v/v CFS supplementation. Karunaratne *et al.* (1990) suggested that competitive growth could inhibit mould growth, as their experiments showed that moulds could not survive beyond four days in the presence of *L. plantarum* and *L. acidophilus* bacterial cells. Additionally, the pH reduction of the environment to around 3.5 - 4.0 in the bacterial cell-free supernatant container was also a significant factor to consider [12].

The inhibition rate was evaluated (Table 2) to compare the antifungal activity of various LAB strains with different fungi.

Table 2. The inhibition rate (%) of cell-free supernatant and biomass of LAB against *A. niger* and *P. oxalicum*

	A. niger		***P. oxalicum***	
	CFS	**Biomass**	**CFS**	**Biomass**
L. paracasei B26010	55.3	47.1	48.2	100.0
L. acidophilus TM	43.5	30.6	62.4	100.0
L. acidophilus VAST	35.3	50.0	84.7	100.0
L. fermentum HA7	49.4	54.1	72.9	100.0
L. rhamnosus GG	52.9	43.5	61.2	100.0

All 5 LAB strains could exhibit inhibition of the growth and development of mould. In general, the CFS and biomass of bacteria inhibited fungi growth at rates ranging from 35.3% to 100%, and the antifungal effect was LAB and fungi strain-dependent. Among LAB-tested strains, *L. acidophilus* VAST showed the best antifungal activity against *P. oxalicum* 20B, whereas this LAB strain showed weakness antifungal effect against *A. niger* CBS 76997.

The differences in the inhibition rates of fungi growth among the tested samples of the same LAB strain indicated that each bacterial strain has distinct mechanisms to inhibit fungi [9]. The metabolism of carbohydrates, proteins, lipids, and amino acids by LAB could produce a variety of antifungal compounds. For example, organic acids such as lactic acid, acetic acid, propionic acid, citric acid, phenyl lactic acid, benzoic acid, and other organic acids produced by LAB have antifungal properties. The lactic acid levels of CFSs showed a positive correlation

with antifungal activity, often synergizing with other organic acids to ultimately enhance antifungal activity. Fatty acids produced by LAB also demonstrated potent antifungal properties. Notably, hexadecenoic acid, oleic acid, hexadecenoic acid, decanoic acid, and lauric acid isolated from LAB confirmed inhibitory effects against *Penicillium*. On the other hand, certain LABs produce hydrogen peroxide, which has been proven to affect the growth and metabolism of foodborne pathogenic bacteria and fungi. As a strong oxidizer, H_2O_2 plunders electrons and molecules of nearby microorganisms and thus sterilizes by destroying protein molecular structure. Due to the absence of catalase production in LAB, H_2O_2 cannot be decomposed and therefore accumulates in the cell, preventing fungal growth. Antifungal peptides also inhibited conidial germination, potentially through the inhibition of germ tube elongation after conidial wall breakdown [9].

6. Conclusion

In conclusion, our study showed the antifungal effect of five lactic acid bacteria including *L. acidophilus* TM*, L. acidophilus* VAST*, L.s fermentum* HA7*, L. rhamnosus* GG *and L. paracasei* B21060 against *Aspergillus niger* CBS 76997 and *Penicillium oxalicum* 20B. The antifungal effect was dependent on LAB and fungi strains. The cell-free supernatant and biomass of LAB could inhibit the growth of tested fungi ranging from 35.3% to 100% and delay the spore-forming process from 2 to 7 days. *L. acidophilus* VAST showed the best antifungal activity against *P. oxalicum* 20B. The results could provide an excellent LAB source for biological preservatives used in feed and food production.

Acknowledgements

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