

UTILIZATION OF LACTIC ACID BACTERIA AND EXTRACELLULAR COMPOUNDS IN BIOLOGICAL CONTROL OF FUNGAL SPECIES

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Abstract: The goal of the present paper was to present the results of the research carried out for selecting efficient bacterial strains against fungal contaminants of vegetal products, to investigate the possibilities to use selected strains and their extracellular compounds in local control of pathogenic or spoilage fungi, as well as the interactions between lactic acid bacteria with antifungal effect and target fungi. Recent research attests the effect of lactic acid bacteria by producing antagonist compounds able to control pathogenic fungi (lactic, acetic, phenyl-lactic, cyclic dipeptides, reuterin, biosurfactants). Lactic acid bacteria selected from various plant materials or traditional Romanian foods were tested for their antagonistic effect on the mycotoxigenic fungal contaminants of fresh and processed fruits or vegetables. In vitro antagonistic activity of 9 lactic acid bacterial strains was assayed against 5 fungal isolates from genera *Penicillium* and *Aspergillus* by double layer method on solid media and by co-cultivation in liquid media. In vivo assay of antifungal effect of two efficient LAB strains as biocontrol agents for apple rot was carried out. Lactic acid bacteria strains LAB 13, LAB 15, LAB 43, LAB 58 presented highly effective antifungal activity, comparably to the reference strains Lpl and Lpa, against pathogenic and spoilage fungal isolates. Antifungal effect of the selected strains revealed by optical microscopy, evidenced structural damages of the hyphae, conidiophores and induced sporulation delays. Among lactic acid bacteria with antifungal activity, the strain LAB 58 presented the highest emulsification ability by releasing biosurfactants with anionic charge, with anti-biofilm effect. Wounded apples treated experimentally with strains LAB 58 and LAB13 and exposed to *Penicillium expansum* spore suspension developed smaller infection spots comparatively with non treated control. Data obtained from in vitro and in vivo assays were similar, confirming the reliability of results and the antifungal activity of LAB strains and their exometabolites, with the possibility to be utilized as biopreservatives for fruits.

Key words: lactic acid bacteria, pathogenic fungi, antagonistic effect, inhibition zone, biopreservation

INTRODUCTION

Fungi, such as those belonging especially to the genera *Penicillium*, *Aspergillus* and *Fusarium*, play a role in damaging various food products stored and subsequently used in human and animal nutrition, by the synthesis of secondary metabolites known as mycotoxins.

In order to reduce the effects of the ingestion of food contaminated with mycotoxins, different strategies applied aimed to avoid contamination of mycotoxins, detoxify food products, inhibit their absorption from the gastrointestinal tract. These strategies involve a variety of physical, chemical, microbiological, biotechnology methods (CUCIUREANU, 2008), but in recent years, particular attention is given to methods of biopreservation and biocontrol (SCHNÜRER AND MAGNUSSON, 2005).

Among the natural biological antagonists, lactic acid bacteria-LAB (with many species as part of the intestinal microflora) have practical applications for the production of fermented foods, enhancement of its quality and as bio-preservatives (CROWLEY et al., 2012),

due to their status of GRAS (Generally Recognized as Safe). Recent research attests the effect of lactic acid bacteria by producing antagonist compounds able to control pathogenic fungi e.g. weak acids (lactic, acetic, phenyl-lactic, propionic) cyclic dipeptides, reuterin, biosurfactants, exopolysaccharides (TRIAS ET AL, 2008; DAHLIE ET AL., 2010).

The aim of the present study, was to investigate the possibilities to use strains of lactic acid bacteria and their extracellular compounds in local control of pathogenic or spoilage fungi, derived from vegetable products used in human nutrition, as well as the interactions between lactic acid bacteria with antifungal effect and various target fungi.

MATERIAL AND METHODS

Test organisms used in this study

Lactic bacteria have been isolated from various plant materials or traditional Romanian food (fermented wheat bran, pickles, sauerkraut). Two collection strains were used as reference strains: *Lactobacillus plantarum* ATCC8014 (Lpl) and *Lactobacillus paracasei* IC13239 (Lpa). Typically, strains were grown in MRS broth at 36^oC.

Potential mycotoxigenic fungal species belonging to genera *Penicillium* and *Aspergillus*, from various plant materials and food products, infected or contaminated (roots and fruits of tomatoes, corn, pickles, assorted pickles, apples, oranges, bread) were also isolated in pure cultures on PDA medium.

Detection of antifungal activity of lactic acid bacteria by double layer method

Antagonistic activity of 9 lactic acid bacterial strains was assayed against 5 fungal isolates from genera *Penicillium* and *Aspergillus*. Discrete spots of each lactic acid bacterial strain suspension were inoculated at constant distances on Petri plates (100mm diameter) containing MRS agar medium and after 48 hours of incubation at 27^oC, plate surface was covered with fungal suspensions mixed with soft PDA. The antifungal activity of lactic acid bacteria was determined by measuring the diameters of the inhibition zones around the spot after 48 hours of incubation.

Interactions between strains of lactic acid bacteria and fungi were examined by optical microscopy and the aspects related to the growth of fungal mycelia, conidiospores forming, development of clear zone around the colony, diffusion of compounds in soft agar have been photographed.

Assay of antifungal activity of LAB strains on fungal growth and biofilm formation

To study the antifungal effect of lactic acid bacteria, 48 hours cultures of each strain (LAB43, LCM58, Lpa, LAB35, LAB15 and LAB13) were inoculated in 100ml flasks over a fungal culture of *Penicillium expansum* (10⁶spores ml⁻¹), in PD broth and monitored for a period of 14 days for biofilm development. Dry weight measurements of fungal biomass (include the extracellular matrix and the cells) were used for direct quantification of influence of lactic acid bacterial strains on the fungal biofilms. Also, the evolution of fungal biofilm was monitored for morphological appearance and sporulation.

Emulsification capacity assay

The emulsification capacity of lactic acid bacterial strains by biosurfactant production, as an extracellular compound was measured. In the case of each strain, the emulsification index (E24) was determined by adding equal quantities of kerosene and cell-free supernatant from MRS broth in test tubes, shaking at high speed by vortex and allowing for 24

hours overnight. Percentage of emulsification index was a ratio between the high of the emulsion layer and the high of total solution (COOPER AND GOLDENBERG, 1987).

Emulsification properties of biosurfactants from lactic bacteria were measured for estimating of their emulsification activity. Thus, the activity of biosurfactants in the process of emulsification was tested and assayed their ability to produce kerosene suspensions with various droplet sizes into aqueous test systems.

Ionic charge of the biosurfactants produced by lactic acid bacteria was observed on 1% agar plate when the precipitation lines appeared between pure compounds with a known ionic charge and the unknown biosurfactant. On both sides of central well with the biosurfactant were placed sodium dodecyl-sulfate and dodecyl-dimethyl-ammonium chloride as anionic and cationic surfactants at a concentration of 0.02M and after application were kept at room temperature for 48h (MEYLHEUC ET AL., 2001).

Ex vivo experiment on apples for antifungal activity of LAB against *P.expansum*

For *ex vivo* experiment, apples cv. Ionatan of Voinesti were surface disinfected with sodium hypochlorite in concentration of 5%, then wounded and sprayed uniformly with the lactic bacterial strains LCM58 and LAB13, grown on MRS broth for 48 hours. After 30 minutes, the apples were infected by spraying with *Penicillium expansum* suspension with a concentration of 10^6 spores ml^{-1} . All assays were carried out in triplicate.

RESULTS AND DISCUSSIONS

Antifungal activity of lactic acid bacteria in double layer assay

Analysis of diameters of the inhibition zones has been carried out in preliminary screening against the fungal species *Aspergillus ochraceus* and *Penicillium digitatum* with different sensitivities to the antifungal action of lactic bacteria. From all strains tested, four had clear areas with diameters above 25 mm (LAB64, LAB15, LAB43, LAB58) compared with those to *Aspergillus ochraceus*, in which clear zones of inhibition of 25 mm were present to 3 strains (LAB15, LAB43, LAB58).

Diameters of the clear zones of inhibition above 25 mm have been obtained against both fungal species for the lactic acid bacteria strains LAB15, LAB43 and LAB58.

For the intensity of antifungal activity of tested lactic acid bacterial strains, some of these were selected for further testing against a number of pathogenic or spoilage fungal species (Figure 1).

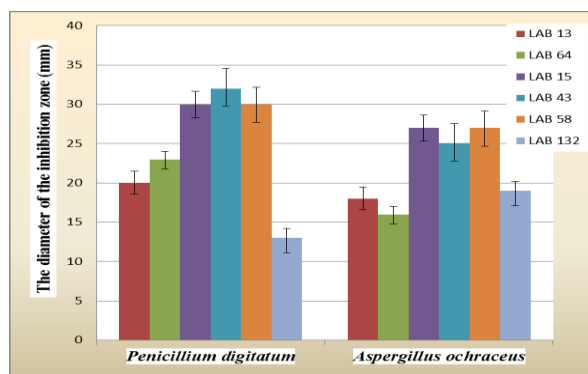


Figure 1 Antifungal activity of six LAB strains against *P. digitatum* and *A. ochraceus*

Aspergillus ochraceus showed sensitivity to the action of selected lactic acid bacteria, the growth and heavy sporulation was inhibited by the lactic acid bacteria strains of LAB 13, LAB64, Lpa, LAB15, LAB43 and Lpl to 5 days.

The inhibitory effect of the strains of lactic acid bacteria LAB13, LAB64, Lpa and LAB43 has remained at the same intensity up to 7 days and partially LAB 15 (Figure 2).

At 3 days, increasing isolate *Aspergillus niger (foetidus)* was intensely inhibited by LAB strains 43, 15 and Lpa. After 5 days, around the spots belonging to the three strains of lactic acid bacteria, was observed the existence of zones of inhibition of sporulation (non sporulating white mycelium) and maintaining a clear zone of antifungal activity for LAB strain 43. No antifungal activity was recorded for all the strains of lactic acid bacteria at day 7 (Figure 2). The inhibition of fungal growth was shown in the case of *Aspergillus flavus* by strains LAB 13, LAB 64, Lpa, LAB 15, LAB 43 and Lpl at 3 days. The strains LAB 13, LAB 15 and Lpa showed even after 5 days clear zones of inhibition, while the antifungal activity of strains LAB 43 and Lpl has been shown only on the sporulation. On day 7, the antifungal activity of strains LAB 13, LAB 15 and Lpa manifested by the presence of non sporulating white mycelia around spots due to inhibition of sporulation (Figure 2).

In the interaction between species *Penicillium digitatum* and lactic acid bacteria, 7 strains (LAB13, LAB64, Lpa, LAB15, LAB43, LAB58, Lpl) showed antifungal activity manifested intense throughout the entire period of monitoring (Figure 2). The observations made on the plates inoculated with eight strains of lactic acid bacteria showed intense inhibitory activity of six strains (LAB13, LAB64, Lpa, LAB15, LAB43 and Lpl) at 3 and 5 days of monitoring, three strains (LAB13, Lpa and LAB15) retain their activity until the seventh day in the tests carried out against *Penicillium expansum* (Figure 2).

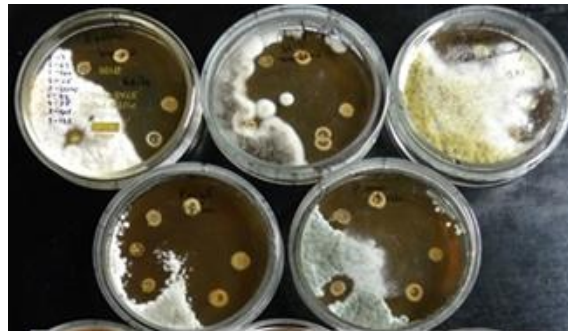


Figure 2. In clockwise order, the spots with lactic acid bacteria strains at 5 days: LAB 13, LAB 64, Lpa, LAB 15, LCM5, LAB 43, LAB 58, Lpl, LAB 132 and Petri plates inoculated with fungal species A- *Aspergillus ochraceus*, B- *Aspergillus niger (foetidus)*, C- *Aspergillus flavus*, D- *Penicillium digitatum*, E- *Penicillium expansum*

Results of *in vitro* assay of antagonistic activity of LAB strains against fungal species are synthesized in the Table 1.

Table 1

Antifungal spectrum of selected strains of lactic acid bacteria

Fungal isolates	Strains of lactic acid bacteria							
	13	15	43	58	64	LCM5	Lpa	Lpl
<i>A.flavus</i> M4	+	+	+	-	+	-	+	+
<i>A.niger</i> M3	-	+	+	±	-	-	±	+
<i>A.ochraceus</i> BZ	+	+	+	-	+	-	+	+
<i>P.expansum</i>	+	+	+	-	+	-	+	+
<i>P.digitatum</i>	+	+	+	+	+	-	+	+

Legend: + = inhibition of fungal growth; ± = weak inhibition of fungal growth; - = lack of inhibition

Research carried out by LAREF AND GUESSAS (2013) showed that 16% of 54 LAB strains from genus *Lactobacillus* produced antifungal compounds active against *Aspergillus*, *Penicillium*, *Fusarium*, *Trichoderma* and *Stemphylium*.16.66% of the strains presented inhibitory activity against *Aspergillus* spp., but no antifungal activity was registered for cell-free supernatant and volatile compounds.

Optical microscopy aspects of interaction between strains of lactic acid bacteria and the fungal isolates

The areas of interaction of selected strains of lactic bacteria and the fungal isolates were examined by optical microscopy to reveal changes caused by antifungal compounds of lactic acid bacteria on structural and morphological characteristics of target fungi.

In the case of LAB 13, the images of the interactions of the extracellular bacterial compounds with fungal mycelium of *Aspergillus ochraceus*, revealed rare hyphae, emptied of content or dry conidiophores deformed and with twisted tips (Figure 3a). Inhibition induced by the lactic bacteria strain LAB 15 to the isolate of *Penicillium digitatum*, manifested, by optical aspects, as a hyphal disorganization (Figure 3b-c) and the appearance of the deformed brushes, atypical and with a retardation of the formation of conidial chains.

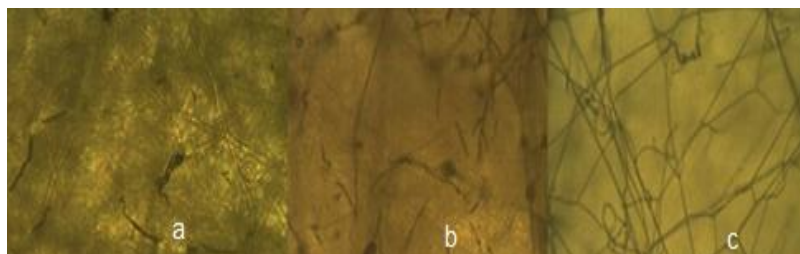


Figure 3. Influence of LAB 13 on a) *Aspergillus ochraceus* isolates (x300) and LAB 15 on b) and c) *Penicillium digitatum* (x300)

Antifungal activity of LAB 43 strain compounds on isolate of *Aspergillus fumigatus* manifested by the deformation of conidiophores in the interaction area, with emptied content, appearance of atypical hyphae with smaller and scarce heads (Figure 4a).

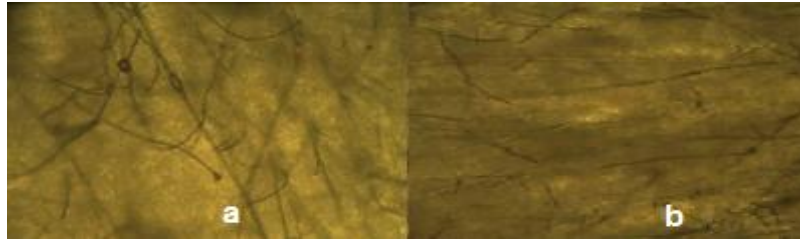


Figure 4. Influence of a) LAB 43 and b) Lpa on *Aspergillus fumigatus* (x150)

The interaction between the collection strain Lpa and of *Aspergillus fumigatus* revealed interrupted hyphae, empty of content, with short conidiophores and with undeveloped heads (Figure 4b)

Also, in the interaction between the same lactic strain and *Penicillium digitatum* appeared vacuolate hyphae, with deformations, the presence of the deformed brushes with scarce conidial chains on the braided hyphae bearing twisted conidiophores, atypical (Figure 5a). In the case of *Penicillium expansum*, these modifications consist in atypical development and absence of the sporulation (Figure 5b).

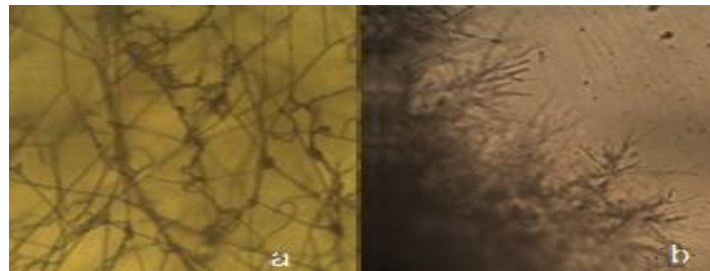


Figure 5. Aspects of optic microscopy of the Lpa influence on a) *Penicillium digitatum* (x300) and b) *Penicillium expansum* (x150)

Similar results from other research reported that fungal growth of *Aspergillus nidulans* was also effected by co-cultivation in liquid media with *Lactobacillus plantarum* MiMAB393 when 36% of the control dry weight was determined and morphological changes induced in mycelia included vacuolization, disturbed hyphal branching and swollen tips (STRÖM, 2005).

Emulsification capacity assay

As an indirect method, emulsification assay was used to screen lactic acid bacteria for biosurfactant production. It was assumed the fact that the cell-free lactic acid bacterial culture filtrates in MRS broth used for evaluation contains biosurfactants which will emulsify the hydrocarbons introduced in the test tubes. Kerosene was used as the hydrophobic substrate in the test. The results obtained revealed that from 8 strains screened (LAB43, LCM5, Lpl, LAB58, LAB35, LAB15, Lpa, LAB13), majority of strains showed intense positive emulsification activity. A weak emulsification activity was found with the strains LCM5 (Figure 6).

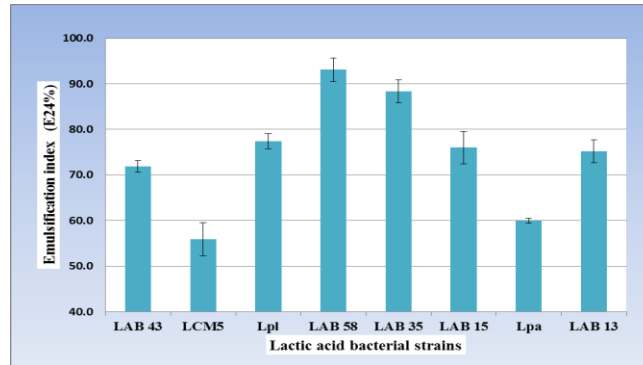


Figure 6. Emulsification activity for lactic acid bacterial strains

The results of emulsification activity analysis showed that bio-surfactant produced by LAB58 strain had the highest emulsification activity (emulsification index of 93,14%) followed by LAB35 strain with emulsification index of 88,37% and by 5 strains (Lpl, LAB15, LAB13, LCM43, Lpa) with E24 index from 77,4%, to 59,8%. The last strain was LCM5 with emulsification index of 55,9%.

The result of emulsification process by bio-surfactant ability to form emulsions in certain conditions was related to the droplet size, knowing the fact that the droplet size is inversely proportional with their activity.

It was observed that isolate LAB58 (figure 7a) and LAB35 showed a great ability to release biosurfactants able to produce droplets with smaller size, acting as a good disperser of the kerosene, in comparing with LCM5 strain which produced large kerosene droplets (Figure 7b). Literature data showed similar values of E24 index for bacterial strains or consortium (up to 93.75%) able to produce biosurfactants.

Biosurfactant properties and the dispersion process could be influenced by genetic potential of the strain for cell wall biochemistry, metabolic state, physical organization of the cell surface biochemistry, binding extracellular molecules more active to surface and which could be involved in antimicrobial activity but, also, by non-biological factors such as: ionic charge of bio-surfactant, temperature, pH, the organic or aqueous phase composition (Sumiardi et al., 2012).

Assay of the ionic charge of biosurfactants released by the strains LAB58 and LCM5 revealed the formation of precipitation lines towards the cationic surfactant dodecyl-dimethyl-ammonium chloride and established the anionic charge of these biosurfactants (Figure 7c)

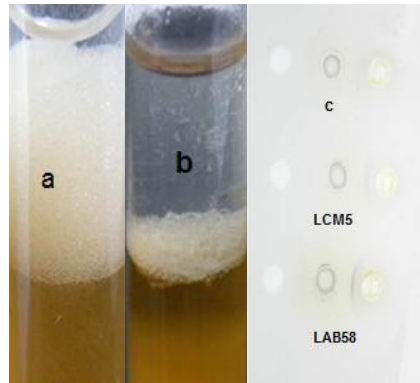


Figure 7. Emulsification pattern of strains a) LAB58 and b) LCM5 and c) the ionic charge of biosurfactants

Growth pattern of lactic acid bacteria biosurfactant producers

In this study, the growth of lactic acid bacteria, producing biosurfactant was assayed to 8 lactic acid bacteria strains (LAB43, LCM5, Lpa, LAB58, Lpl, LAB13, LAB64 and LAB132), results indicating that didn't exist different growing patterns.

Figure 8 presents the values of optical density for each strain measured by spectrophotometry at 660nm.

The results of measurements of growth patterns of lactic acid bacterial showed an exponential phase after 24 hours of incubation, followed by a stationary phase up to 120 hours and a tendency to decrease in the decline phase between 120-170hours.

During stationary phase, increasing in the cell sizes slowed and the susceptibility to physic-chemical factors increased.

In the decline phase, the growth rate decreased, the number of living cells declined, it began to manifest unequal growth with abnormal cells or with aberrations as a result of waste products accumulation, pH changes and lack of nutrients.

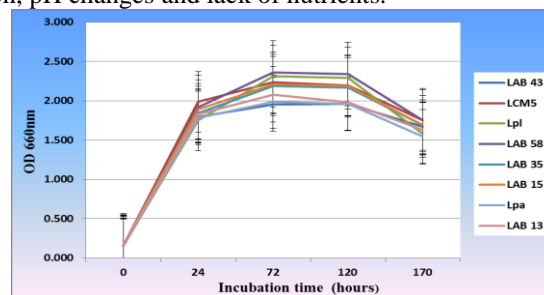


Figure 8. The growth of lactic acid strains during incubation time

Assay of antifungal activity of LAB on fungal growth and biofilm formation

Effect of introducing of strains and extracellular compounds of lactic acid bacteria on the fungal biofilm developed by *Penicillium expansum* was analyzed directly, by way of quantitative changes occurring in the biomass accumulation processes, in conditions of simultaneous development of both species.

Biomass accumulations are shown in the figure 9 and data analysis revealed that the strain LAB13 influenced most powerful the processes involved in the perforation of fungal biofilm, causing an inhibition of growth and of fungal mycelia development in the tested isolate. Also, in terms of the intensity of the inhibition, the strains LAB43, LAB15, LAB58 and Lpa had allowed higher fungal biomass accumulations compared with strain LAB13 but smaller compared to the control, which lacked a strain of lactic bacteria inoculated in fungal growth medium. Fungal biomass accumulation was higher at the inoculation with LCM5 strain and it represented 53.71% of those obtained at control variant, aspect that causes us to consider that it was the weakest from the lactic bacteria strains tested for the anti-biofilm activity.

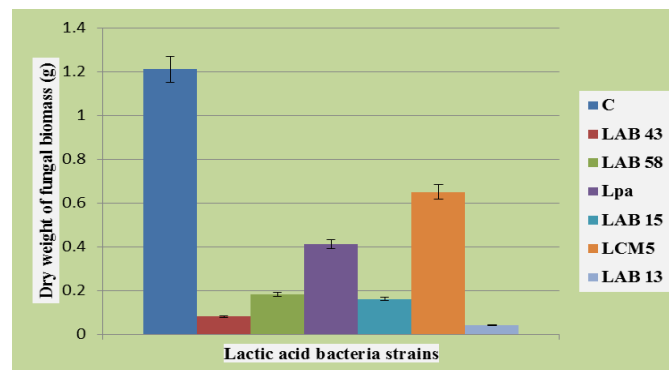


Figure 9. Inhibition of fungal growth and biofilm formation by LAB strains

Differences of influencing of the LAB strains (LAB58 and LAB13), both considered active in the anti-biofilm activity, were further analyzed by images of the fungal biofilms of *Penicillium expansum* and on the apples treated *in vivo* with these lactic acid bacteria and which subsequently were infected with the same fungal specie.

The images revealed, in first case, morphologic changes of biofilm induced by the presence of the lactic bacterial strains (Figure 10b and 10c), in comparing with fungal biofilm at control variant (Figure 10a) and also, the different sporulation capacity of the fungus as influenced by LAB strains activity.

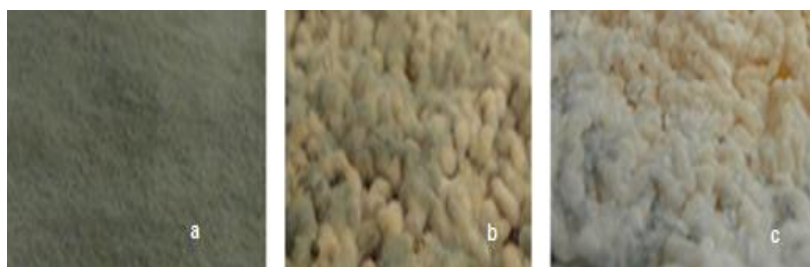


Figure 10. a) Biofilm (*Penicillium expansum*), b) biofilm + LAB58, c) biofilm + LAB13

Similar results were reported for extracellular LAB extracts on different pathways of bacterial biofilm development (El-Deeb et al., 2015). The same *Lactobacillus acidophilus* strain inhibited over 59% biofilm formation in *Shigella* and one *Klebsiella* strain but not in two

other *Klebsiella* strains.

Antifungal activity of LAB against *P.expansum* (ex vivo experiment on apples)

When tested *ex vivo*, the effect of applying the lactic bacteria strains and their extracellular compounds appeared more obvious. Thus, the apples wounded, treated with lactic acid bacteria and subsequently infected with *P. expansum*, resisted to the phytopatogen attack better, the average diameter of infection from 6 wounds on each apple were of 29.64mm and of 32.47mm respectively, for the two bacterial strains, much lower than diameter of infection spots from untreated control (41.34mm), and which began spots confluence after only 7 days from fungal infection (Figure 11).



Figure 11. Aspects of wounded apples infected with *Penicillium expansum* and treated with LAB58 and LAB13 strains

The results obtained for *ex vivo* experiment were similar to those obtained by Rouse et al. (2007) in *ex vivo* experiment on growth medium supplemented with apple extract, where inhibition of spoilage fungus *Penicillium expansum* was induced by antifungal compounds produced by a LAB strain of *Pediococcus pentosaceus* (with inhibition zones over 10mm diameter).

The results of the three assays carried out *in vitro* on solid and liquid media and *in vivo* on apples were comparable, confirming the effectiveness of antifungal activity of tested LAB strains.

The results obtained by ADEBAYO AND ADERIYE (2010) when assayed the antifungal activity of LAB strains from genus *Lactobacillus* against *Penicillium citrinum* appeared to be similar as pattern of fungal sensitivity. Authors evidenced a higher percent of growth inhibition in liquid media than on agar plates and explained this by a greater diffusion of antifungal compounds in liquid media and a higher contact with fungal spores than on solid media. They observed an inhibition level over 25% for 10 of 17 LAB strains and attributed this inhibitory effect to bacteriocins.

CONCLUSIONS

Lactic acid bacteria strains LAB 13, LAB 15, LAB 43, LAB 58 presented highly effective antifungal activity, comparably to the reference strains Lpl and Lpa, for pathogenic and spoilage fungal isolates.

Antifungal effect of the selected strains was revealed by optical microscopy, evidencing structural damages of the hyphae, conidiophores and sporulation delays.

Among lactic acid bacteria with antifungal activity, the strain LAB58 presented the highest emulsification ability by releasing biosurfactants with anionic charge, with antibiofilm effect.

Data obtained from *in vitro* and *in vivo* assays were similar, confirming the reliability of results and the antifungal activity of LAB strains and their exometabolites, with the possibility of using as biopreservatives for fruits.

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