Antifungal activity of lactic acid bacteria *in vitro* and *in situ* as bio-preservative

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Abstract: The rising concern over food safety and spoilage caused by fungal contamination has highlighted the need for natural and effective bio-preservatives. This study aimed to isolate and identify lactic acid bacteria (LAB) from dairy products with antifungal properties and to characterize their bioactive compounds for potential use as bio-preservatives. Out of 208 isolates from the dairy products, from 80 LAB isolates, only four demonstrated the ability to inhibit the growth of Aspergillus flavus. The scope of the study was then expanded to assess the antifungal effects of these isolates against a broader range of fungal strains, including *Aspergillus niger*, *Mucor* sp., *Rhizopus stolonifer*, *Alternaria citri*, *Aspergillus niger* ch01, and *Aspergillus flavus* ch02. LAB isolate "B 50" exhibited the strongest antifungal activity, effectively inhibiting both fungal spore germination and mycelial growth. Molecular identification confirmed "B 50" as *Lacticaseibacillus rhamnosus*. The shelf life of cheese slices with added supernatant of *L. rhamnosus* was longer than control. The cell-free supernatants of selected *L. rhamnosus* was identified and quantified as formic, lactic, acetic and succinic acids at concentration of 1.541, 18.535, 7.113 and 7.154 mg ml^{-1}, respectively. The integration of both *in vitro* and *in situ* screening experiments allowed to select the highly significant strain *L. rhamnosus* as a target to fungal growths of selected fungi, with antifungal activities as food bio-preservatives.

Keywords: Antifungal activity, bio-preservative, *in vitro* and *in situ*, *L. rhamnosus*, lactic acid bacteria

INTRODUCTION

FAO studies indicate that about one-third of food amount prepared for human consumption worldwide is lost or spoiled annually (Salas *et al*., 2017). Fungi play a major role in the corruption of many foods due to their ability to grow in difficult environmental conditions. Also, fungi cause many serious health problems for humans and animals due to the release of mycotoxins (Lowe and Arendt, 2004). Regarding food quality, fungal presence and growth can also lead to visual, texture and organoleptic defects. The fungal growth of *Aspergillus* and *Penicillium* are the main spoilage of dairy products inducing great economic losses (Gerez *et al*., 2009; Garnier *et al*., 2020). *Aspergillus niger* considered one of the most important fungi that contaminates food and dairy products. Moreover, some fungal genera have the ability to produce secondary metabolites which have negative impact on humans and animals such as *Alternaria, Penicillium and Fusarium* (Salas *et al*. 2017). These fungi are most resistant that can grow in low temperature and pH. Dairy products such as cheese, fermented milks and yoghurt, which have a great economic importance in food industry, are subject to contamination with fungi (Delavenne *et al*., 2012). However, synthetic fungicides generally used to prevent fungi in food. There is an increasing interest during the recent years to limit the use of chemical compounds and use microorganisms as natural bio-preservation. Beyond this negative impact of using chemical preservatives on the food quality, many studies have successfully tested LAB as potential antifungal cultures. Furthermore, many LAB have been regarded as "Green preservatives" because their role to limit and inhibit fungal growth in foods. The main microorganisms were widely applied in dairy products are *L. rhamnosus*, *L. plantarum* and *L. paracasei* (Bazukyan *et al*., 2018; Ouiddir *et al*., 2019; Nasr and Abd-Alhalim, 2024). The antifungal capacity of LAB is due to their metabolites that include organic acids, diacetyl, fatty acids, bacteriocins, low molecular weight compounds, cyclic dipeptides, exopolysaccharides and reuterin (Fernandez *et al*., 2017; Luz *et al*., 2017; Ibrahim *et al*., 2021; Guimarães and Venâncio, 2022; Iosca *et al*., 2022; Liu *et al*., 2022). Moreover, these large spectrums of compounds could have antagonistic activity towards pathogenic microorganisms. In this context, we targeted strains with antifungal activity *in vitro* and *in situ* as actual food for accurate selection of isolates with the potential to appear and/or produce antifungal compounds as a vital means of food bio-preservation rather than chemical preservatives.

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MATERIALS AND METHODS

I. Isolation of microorganisms from milk and dairy products:

I.1 Samples: About 50 samples of raw and colostrum milks, yoghurt, rayeb milk, Damietta cheese, Karish cheese, Ras cheese, Talaga cheese and Istanbuly cheese were collected from Ismailia, Port Said and EL-Sharkia Governorates, Egypt. Five samples of cheese were collected from Egyptian's supermarket for fungi isolation.

I.2.a Isolation of lactic acid bacteria: Sample (1 ml or gram) was taken aseptically and homogenized in 9 ml of sterile saline solution, except cheese samples which were taken out by sterile knife under aseptic conditions and ground in previously sterilized mortar with 1 ml of sterilized sodium citrate solution (20%, w/v) and 8 ml sterile saline solution (0.85%, w/v NaCl) previously warmed to 37 ºC to give a dilution of 10^{-1}. Serial dilutions were done then 1 ml of each dilution was cultured in Petri dishes. About 10 ml of MRS agar medium (De Man *et al*., 1960) was poured, solidify and the plates were incubated at 37 ºC for 48 h. Single colony was selected, streaked 3 times on MRS medium and incubated at 37 ºC for 48 h. Purified cultures were inoculated in MRS broth medium and incubated at 37 ºC for 48 h to obtain a good growth and kept at -18 ºC in MRS broth medium containing (20%, v/v) glycerol until used.

I.2.b Preliminary identification of LAB isolates: Bacterial isolates which appeared antifungal activity were examined for Gram, spore stain (Pelczar and Chain, 1977) and catalase activity (Mac Faddin, 1977).

I.3.a Isolation of fungi: Fungi were collected from the outer surfaces of the cheese with a sterile knife. The fungi were isolated by serial dilution using standard plate count (SPC) agar medium supplemented with antibiotics after incubation at 27 ºC for 4 days (Marshall, 1992). Single fungi colony was collected and streaked 3 times. Purified fungi cultures were inoculated on SPC slant agar medium and incubated at 27 ºC for 4 days and kept at 4 ºC until used.

I.3.b Fungal strains: *Aspregillus flavus* (target) was obtained from Mycotoxin Laboratory, National Research Centre, Cairo, Egypt. *Aspregillus niger* was obtained from international mycological institute, ferry lane, Kew, surrey, Tw 93AF, UK. *Mucor* sp., *Rhizopus stolonifera* and *Alternaria citri* were obtained from Botany Department, Faculty of Science, Suez Canal University, Ismailia, Egypt.

I.3.c Preparation of fungal spore suspensions: Fungal culture was prepared by inoculation of fungus on the surface of SPC agar slants and incubating at 27 ºC for 7 days for each spore suspensions. The spores were harvested with sterile phosphate diluted buffer plus 0.05% tween 80 (pH 7.20). The buffer solution was used to collect fungal spores from the slant. The fungal spore suspension was purified by filtration twice through several layers of sterile damp cheese cloth to separate fungal spores from hypha fragments (Osman,1999).

I.3.d Preparation of fungal mycelium block: Fungal culture was prepared by inoculating 1 ml of fungal spores on the surface of SPC agar plates and incubated at 27 ºC for 48 h. A block of the fungal culture was cut by 5 mm sterile cork borer and used for inoculation the media or cheese to detect the fungal growth and spore onset (Osman, 1999).

II.1 Screening for antifungal activity *in vitro*: The antifungal activity of LAB isolates was examined on the surface of MRS agar medium by using of fungal spore suspension and fungal mycelium block.

II.2.a Screening of antifungal activity by fungal spore suspension: LAB isolates were inoculated into 10 ml of sterile MRS broth medium and incubated at 37 °C for 48 h. A loop of the bacterial isolate was inoculated in 2 cm lines on the surface of MRS agar medium plates and allowed to grow at 37 ºC for 48 h. The plates were overlaid with sterile SPC agar medium, solidify and inoculated with 100 µl of fungal spore suspension in the center (10^5 spores ml^{-1}). The plates were incubated at 27 ºC for 14 days and examined daily for the fungal growth and spore onset. Control plates of MRS agar medium overlaid with sterile SPC agar medium were used by inoculating the plates by 100 µl of the fungal spore suspension (Kivanc *et al*., 2014).

II.2.b Screening of the antifungal activity by fungal mycelium block: LAB isolates were inoculated into 10 ml of sterile MRS broth medium and incubated at 37 °C for 48 h. A loop of the bacterial isolate inoculated in 2 cm lines on the surface of MRS agar medium plates and allowed to grow at 37 ºC for 48 h. Block of the fungal culture grown on SPC agar medium after

incubation at 27 for 4 days was cut by 5 mm sterile cork borer. The fungal block was placed between the two parallel lines of activated isolate grown on MRS agar medium. The plates were incubated at 27 ºC for 14 days and examined for the fungal growth and spore onset. Control plates of MRS agar medium were used by inoculating the plates by fungal mycelium block (Osman, 1999).

II.3 Identification of selected LAB by 16S rRNA sequencing and fungi by 18S rRNA:

II.3.a LAB isolate: Selected LAB isolate was inoculated in 10 ml MRS broth medium and incubated at 37 °C for 48 h. MRS liquid culture (1 ml) was centrifuged (microcentrifuge, Minispin, Eppendorf, Germany) at 1000 ×*g* at 4 ºC for 15 min to obtain the pellet of isolate (Rossi *et al*., 2012).

II.3.b Fungal isolates: Once fungal strains had formed colonies, a sterile needle was used to transfer a small amount into an Eppendorf tube and phosphate diluted buffer (500 µl) was added. The Eppendorf tube was centrifuged at 1000 ×*g* for 15 min for fungal pellet collection (Wu *et al*., 2001).

II.3.c Identification of LAB and fungi: The selected LAB and fungal isolates were identified by sequencing analysis of 16S and 18S rRNA, respectively. The isolates were subjected for the genomic DNA extraction using using DNeasy Tissue Mini Kit (Qiagen, Valencia, CA). The 16S gene was performed using primers pairs 27F (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492R (5'-GGT TAC CTT GTT ACG ACT T-3') (McCabe *et al*., 1999), in thermal cycler (MJ research thermal cycler, USA) under the following conditions: (1) initial denaturing step at 95 ºC/ 3 min, (2) 35 cycles of denaturation (95 ºC/ 30 s), annealing (50 ºC/ 30 s) and extension (72 ºC/ 90 s) and (3) final extension at 72ºC/ 5 min. The 18S gene was also amplified with primers pairs ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') (Yonemori *et al*., 2002). The resulted sequences were trimmed and assembled in Geneious software (Biomatters). Consequently, the trimmed sequences were identified by search in basic local alignment search tool BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) in GenBank.

II.4 Antifungal activity of LAB against different fungal spores in liquid medium: LAB isolate was inoculated in 50 ml MRS broth medium and incubated at 37 °C for 48 h. The flasks were inoculated with *Aspregillus flavus*, *Aspregillus niger*, *Mucor* sp., *Rhizopus stolonifera*, *Alternaria citri* or 2 isolated fungi (ch01 and ch02) using 100 µl of spore's suspension (10^5 spores ml^{-1}) and incubated at 27 ºC up to 14 days to detect fungal growth and spore onset (Ayesh and Osman, 2003).

II.5 Detection of optimum time for antifungal activity: Selected LAB isolate was inoculated in 50 ml MRS broth medium and incubated at 37 °C for 24, 48, and 72 h. The bacterial growth was stopped by adding 2% antibiotic solution (Marshall, 1992). The flasks were inoculated with *Aspregillus flavus* using 100 µl of spores suspension (10^5 spores ml^{-1}) or fungal mycelium block and incubated at 27 ºC up to 14 days to detect fungal growth and spore onset (Ayesh and Osman, 2003).

II.6 The effect of pH on the antifungal activity: LAB isolate was inoculated in 50 ml MRS broth medium and incubated at 37 °C for 48 h. The bacterial growth was stopped by adding 2 % antibiotic solution. The pH of bacterial culture was adjusted with sterile 1 M NaOH to pH 6.4 and inoculated with 100 µl of spores suspension (10^5 spores ml^{-1}) or fungal blocks and incubated at 27 °C for 14 days to detect fungal mycelium growth and spore onset (Osman, 2004).

II.7 Mycelial dry weight: In this experiment Whatman no.1 papers were dried to a constant weight and weighted before filtrate the mycelia mats of fungi grown in MRS broth medium at 27 ºC for 14 days. The filter paper plus mycelia were then dried (MLW-VEB, WST 3010) at 65 ºC for 24 h and transferred to a desiccator. The net of mycelia dry weight per mg ml^{-1} were obtained by subtraction of dried control filter paper from the weight of the experimental mycelia and filter paper (Ayesh and Osman, 2003).

II.8 Quantification of organic acids by HPLC

II.8.a Bacterial supernatant preparation: LAB isolate was inoculated in 50 ml MRS broth medium and incubated at 37 ºC for 48 h. The cultures were centrifuged (IEC-7000, USA) at 2465 × *g* for 30 min at 4 ºC. The supernatant was transferred to a sterile tube kept at -18 ºC until used.

II.8.b Quantification of organic acids compounds: The organic acids present in cell-free supernatants (CFS) after 48 h were determined by HPLC (Agilent HPLC 1260 series model, U.S.), equipped with a quaternary pump and diode array detector was monitored at 210 nm. Formic, lactic, acetic, citric, succinic and propionic acids were determined using an Eclipse AQ-C18 HP column (4.6 mm x 150 mm i.d., 3 µm) under the following conditions:

mobile phase (0.005N sulfuric acid); flow rate 0-4.5 min (0.8ml/min);4.5-4.7 min (1 ml/min); 4.7-4.71 min (1 ml/min); 4.71-8.8(1.2 ml/min); 8.8-9(1.3 ml/min); 9-23(1.3 ml/min); 23-25(0.8 ml/min), respectively, and temperature of column set to was 55 ºC.

II.9 Antifungal activity *in situ* screening: Processed cheese slice (Teama, Egypt) was divided in 4 pieces and distributed in 4 Petri dishes which containing a sterile paper soaked with sterile water. LAB supernatant 800 µl was sprayed on the surface of cheese slice and left to dry at room temperature. The middle of cheese slices were inoculated with 20 µl of fungal spores suspension (10^5 spores ml^{-1}) or fungal mycelium blocks. The plates were incubated at 27 ºC. The fungal growth and spore onset were daily detected up to 14 days (Le Lay *et al*., 2016b).

RESULTS AND DISCUSSION

I. Isolation of LAB from milk and dairy products: A total of 208 bacterial isolates were isolated from milk and dairy products. The preliminary screening of the pure colonies showed that 80 isolates were Gram positive, non-spore forming, catalase negative and bacilli and cocci-shaped. These isolates were identified as LAB (Panebianco and Caridi, 2021). All selected LAB isolates were tested for their antifungal properties against the fungal spore suspension of the target fungus (*Aspregillus flavus*). Only 4 LAB isolates were confirmed to have antifungal activity against the growth of *A. flavus*. These results are in agreement with Al-shammari and Majeed (2016) ; Le Lay *et al*. (2016b), in which their LAB isolates (*Leuconostoc citreum*, *Lactobacillus sakei*, *Lactobacillus plantarum*, *Lactobacillus spicheri*, *Lactobacillus reuteri, Lactobacillus brevis*, *Lactobacillus fermentum*, *Lactobacillus* sp. and *Lactococcus* sp.) exhibited antifungal activity against different fungi (*Cladosporium sphaerospermum*, *Wallemia sebi*, *Eurotium repens*, *Aspergillus niger*, *Penicillium corylophilum*, *Fusarium oxysporum*, *Phytophthora infestans*, *Pythium ultimum* and *Alternaria* sp.).

II. Identification of the isolated fungi: BLAST results identified the fungal isolates ch01 and ch02 isolated (from Ras cheese) as *Aspergillus niger* and *Aspergillus flavus*, respectively. *A. niger* and *A. flavus* were isolated from Ras cheese by El-Fadaly *et al*. (2015); Elramly *et al*. (2019); Moneeb *et al*. (2022).

III. Antifungal activity assays *in vitro:-*

III.1 Antifungal activity of LAB isolates against different fungal spores: The selected four LAB isolates were tested against seven fungal spore suspensions which included, *A. niger*, *A. flavus, Mucor* sp.*, Rhizopus stolonifer, Alternaria citri, A. niger* ch01 and *A. flavus* ch02 (Table 1 and Fig. 1). The growth and spore onset of all target fungi grown on control SPC agar medium were after 2-3 and 2-5 days, respectively. All LAB isolates were preventing the growth and spore formation of *A. flavus* and *Alternaria citri*. LAB isolate number B88 inhibited the growth and spore formation of all target fungi while, delaying the growth and spore onset (3 and 4 days, respectively) of *A. niger* ch01. LAB isolate number B80 was delayed the growth and spore onset of *A. niger* while, delayed the spore onset of *Mucor* sp., *Rhizopus stolonifer, A. niger* ch01 and *A. flavus* ch02 without affecting in the growth onset of these fungi. LAB isolate number B103 inhibited the growth and spore formation of *A. flavus, Mucor* sp., *Rhizopus stolonifer* and *A. niger* ch01 while, delayed the growth and spore onset of *A. niger* and *A. flavus* ch02. LAB isolate number B50 inhibited the growth and spore formation of all target fungi. These results indicated that LAB isolate number B50 showed antifungal activity against all target fungi. These results are in agreement with Cortés-Zavaleta *et al*. (2014), who found that *L. rhamnosus* had antifungal activity against some food spoilage molds, including *A. flavus.* In similar studies by Sadeghi *et al*. (2016) and Muhialdin *et al*. (2018), lactic acid strains inhibited *A. flavus* and *A. niger* growth, while others LAB strains have failed to inhibit the growth of *A. niger* in fermented food (Iosca *et al*., 2022). Also, the results are in agreement with those by Cosentino *et al*. (2018), who indicated uninhibition of some LAB (*L. plantarum* 1B3M*, L. plantarum* 10B3M, *L. paracasei* 1A6M, and *L. brevis*) against the growth of *Mucor recurvus*. While, *L. rhamnosus* MDC 9661 had inhibitory activity against *Mucor plumbeus* growth (Bazukyan *et al*., 2018). Prema *et al*. (2010) reported that *L. plantarum* has the ability to inhibit the growth of *Rhizopus stolonifer* and *A. fumigatus*. Many of LAB isolates showed inhibitory activity against *Alternaria alternate* (Riolo *et al*., 2023). At the same time, *L. plantarum*, *L. paracasei* and *L. brevis* showed no antifungal activity against *Alternaria alternata* (Cosentino *et al*., 2018). Lavermicocca *et al*. (2000) reported that *L. plantarum*, was able to inhibit *A. niger*. Fernandez *et al*. (2017) found that

using a single strain *L. rhamnosus* was able to delay fungal growth for 4 days in skim milk agar medium, while a combination with other LAB strains inhibited the fungal growth for 21 days. The difference in the antifungal activity of LAB strains may be due to their metabolites (Souza *et al*., 2023).

Table (1) Antifungal activity of LAB isolates against different fungal spores and mycelia on agar medium after incubation at 27 ºC for 14 days

LAB isolates	Control		B 88		B 80		B103		B50	
	Fungal growth and sporulation onset per days									
Target fungi	Growth	Spore	Growth	Spore	Growth	Spore	Growth	Spore	Growth	Spore
	Inoculation by fungal spores									
A. flavus	2	2	-	-	-	-	-	-	-	-
A. niger	2	3	-	-	3	4	3	4	-	-
Mucor sp.	2	3	-	-	2	4	-	-	-	-
Rhizopus stolonifer	2	3	-	-	2	4	-	-	-	-
Alternaria citri	3	5	-	-	-	-	-	-	-	-
A. niger ch01	2	3	3	4	2	4	-	-	-	-
A. flavus ch02	2	2	-	-	2	4	3	4	-	-
	Inoculation by fungal mycelia									
A. flavus	2	2	3	4	3	4	3	4	4	5
A. niger	2	3	3	4	3	4	3	4	-	-
Mucor sp.	2	3	2	3	2	3	2	3	3	4
Rhizopus stolonifer	2	3	2	3	2	3	2	3	-	-
Alternaria citri	2	4	-	-	-	-	-	-	-	-
A. niger ch01	2	4	2	4	2	4	2	3	7	8
A. flavus ch02	2	2	3	4	3	4	3	4	-	-

(-) No growth or sporulation until 14 days

III.2 Antifungal activity of LAB isolates against different fungal mycelia: The selected four LAB isolates were tested against seven fungal mycelia which included *A. niger*, *A. flavus, Mucor* sp., *Rhizopus stolonifer, Alternaria citri, A. niger* ch01 and *A. flavus* ch02 (Table 1 and Fig. 2). The growth and spore onset of all target fungi on control MRS agar medium were after 2 and 2-4 days, respectively. All LAB isolates prevented the growth and spore formation of *Alternaria citri*. Isolates numbers B88, B80 and B103 of LAB uninhibited the growth and spore formation of *Mucor* sp., *Rhizopus stolonifer* and *A. niger* ch01 while, delayed the growth and spore onset of *A. flavus*, *A. niger* and *A. flavus* ch02 by 3 and 4 days, respectively compared with control. LAB isolate number B50 inhibited the growth and spore formation of *A. niger*, *Rhizopus stolonifer*, *Alternaria citri* and *A. flavus* ch02 while, delayed the growth and spore onset of *A. flavus* by 4 and 5 days, respectively, *Mucor* sp. by 3 and 4 days, respectively and *A. niger* ch01 by 7 and 8 days, respectively compared to control. In a similar study, Al-Shammari and Majeed (2016) reported that *L. fermentum* and *L. reuteri* appeared antifungal activity against *Alternaria* sp. In addition, Osman (1999) found that *Brevibacterium linens* showed varied antifungal activity against the mycelia of *A. flavus, A. niger*, *Mucor* sp., *Rhizopus stolonifer* and *Alternaria citri.* LAB isolate number B50 inhibited the growth and spore formation of *A. niger*, *Rhizopus stolonifer*, *Alternaria citri* and *A. flavus* ch02 mycelia. Therefore, isolate B50 was selected as antifungal LAB strain for the next experiments due to its inhibition of growth and spore formation for all target spore and most mycelium fungi. Maybe these differences in inhibition effects on spores and mycelia are due to the different antifungal mechanisms of organic acids, which could disrupt the microbial spore of the cell membrane by causing collapse of the phospholipid bilayer through depolarizing the transmembrane electrical potential. Moreover, these organic acids in cell-free supernatant (CFS) could also damage the permeability of the mycelia

membrane through leakage of K^+ (Ma *et al*., 2024).

III.3 Identification of the selected LAB isolate: The resultant sequence of B50 was compared with the sequences of LAB strains present in the GenBank of NCBI database. B50 isolate was identified as *Lacticaseibacillus rhamnosus*. As mentioned before LAB isolate number B50 (*L. rhamnosus*) was isolated from dairy product. *L. rhamnosus* was also isolated by many researchers (Ayad *et al*., 2006; Delavenne *et al*., 2013; Bazukyan *et al*., 2018; Matevosyan *et al*., 2019; Akhtach *et al*., 2021; Tahoun *et al*., 2021; Nasr and Abd-Alhalim, 2024), from Ras cheese and other dairy products.

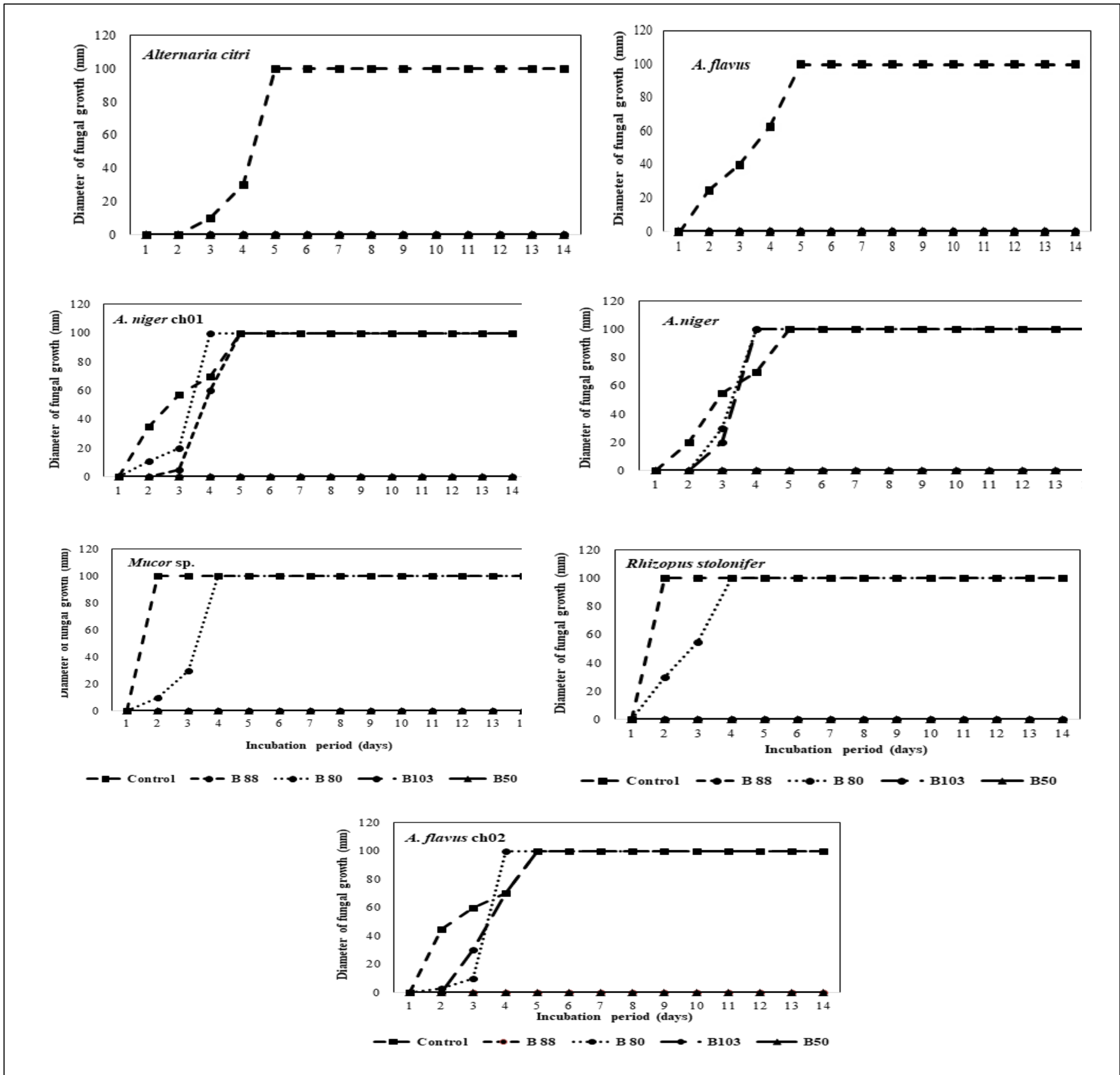

Fig. (1): Antifungal activity of LAB isolates against the growth of different fungal spores on SPC agar medium after incubation at 27 ºC for 14 days

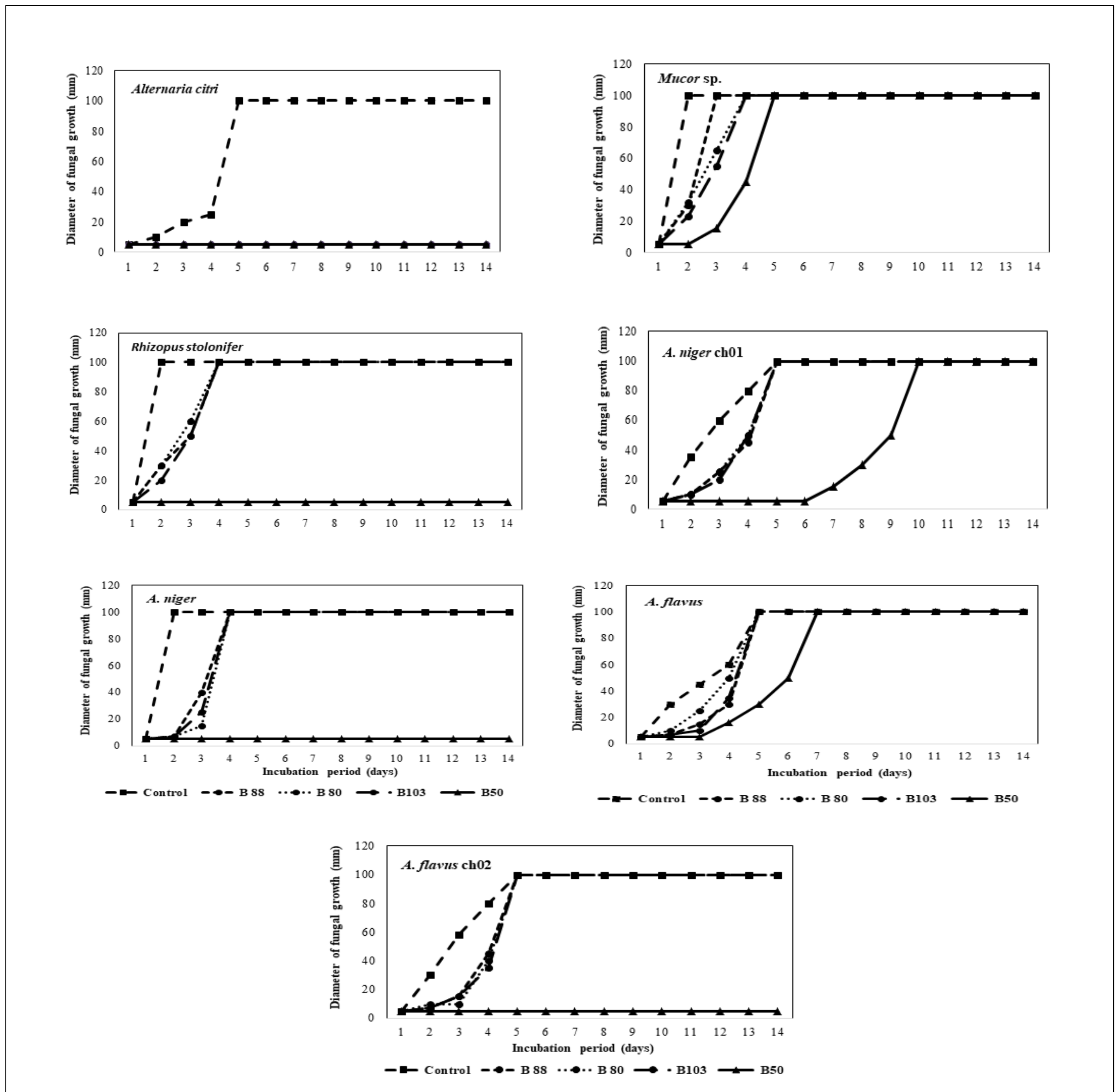

Fig. (2): Antifungal activity of LAB isolates against the growth of different fungal mycelia on MRS agar medium after incubation at 27 ºC for 14 days

III.5 The optimum time of antifungal activity: The inhibition of the growth and spore onset of *A. flavus* in MRS broth medium (control) when inoculated with the fungal spores was 3 and 5 days, respectively. While, when inoculated by mycelium, the growth and spore onset were 2 and 4 days, respectively. *L. rhamnosus* grown in MRS broth medium at 37 ºC for 1, 2 and 3 days inhibited the growth and spore formation of *A. flavus* (inoculated by spores or mycelium). The inhibition of the growth and spore formation of *A. flavus* may be due to production of some antifungal metabolites by *L. rhamnosus* such as organic acids (acetic, lactic, propionic, and phenylacetic acids), rutrin, fatty acids hydrogen peroxide, cyclic dipeptides and proteinaceous compounds (Ouiddir *et al*., 2019; Cosentino *et al*., 2018). The antifungal activity appeared on the first day and increased gradually until the third day. These results are in agreement with Parappilly *et al*. (2022) who found a significant

effect on the inhibition of bacterial isolates against *A. flavus* when using different incubation times for bacteria due to increased organic acids with the increasing the bacterial age.

III.6 Antifungal activity of *L. rhamnosus* against different fungal spores in liquid medium: *L. rhamnosus* was able to inhibit the growth of all fungal spores in MRS broth culture including *A. flavus*, *A. niger*, *Mucor* sp., *Rhizopus stolonifer*, *Alternaria citri*, *A. niger* ch01 and *A. flavus* ch02. The antifungal activity of LAB may be due to the production of some different compounds, such as exopolysaccharides (EPS), volatile and organic acids, hydrogen peroxide, various lipids and bacteriocins (Matevosyan *et al*., 2019; Iosca *et al*., 2022). In this context, Cortés-Zavaleta *et al*. (2014) showed antifungal activity of LAB such as *Lactobacillus acidophilus* against molds. In other study by Osman (2004) the antifungal activity of *Brevibacterium linens* cell-free supernatant against *A. flavus* was detected.

III.7 Antifungal activity of *L. rhamnosus* after pH adjustment: The growth and spore formation of *A. flavus* grown in MRS broth medium (control, pH 6.4) when inoculated by fungal spores were 3 and 5 days, respectively with final pH (after 14 days) was 7.49 (Table 2). While, the fungal growth and spore formation when inoculated by fungal mycelium were 2 and 4 days, respectively with final pH 9.11 (after 14 days). Mycelium dry weight of *A. flavus* (control) when inoculated with spores and mycelium were 9.198 and 11.946 mg ml^{-1}, respectively. *L. rhamnosus* (at pH 3.6) inhibit the growth and spore formation of *A. flavus* (inoculated by spore or mycelium). When the pH of *L. rhamnosus* broth culture adjusted to pH 6.4, the growth of *A. flavus* (inoculated by spore or mycelium) was 5 days without spore formation (with final pH 8.103 and 8.895, respectively). While, mycelium dry weight of *A. flavus* when inoculated by spores and mycelium in *L. rhamnosus* broth culture were 8.604 and 8.9 mg ml^{-1}, respectively. Which mean that mycelium dry weight of *A. flavus* decreased by 6.46 and 25.50 %, respectively. Le Lay *et al*. (2016a), mentioned that the antifungal activity appeared by LAB may be due to the organic acids production. Cortés-Zavaleta *et al*. (2014) reported a decrease in antifungal activity when adjusted the pH to 6.5. Another study reported that the antifungal activity was stable under different pH values, suggesting that the activity was not related to acids (Sedaghat *et al*., 2016).

III.8 Quantification of organic acids produced by *L. rhamnosus*: HPLC analysis of the cell-free supernatant for *L. rhamnosus* grown in MRS broth medium at 37 °C for 48 h revealed that the strain produced formic, lactic, acetic and succinic acids at concentration of 1.541, 18.535, 7.113 and 7.154 mg ml^{-1}, respectively (Table 3). These results are in agreement with Lynch *et al*. (2014), who reported that the antifungal activity of LAB may be due to the combined effect of different metabolic compounds. However, Gerez *et al*. (2009) found that propionic acid was more highly effective than acetic acid while, phenyllactic acid was more highly effective than lactic acid on the growth of *A. niger*.

Table (2) Effect of adjustment the pH *L. rhamnosus* grown in MRS broth medium on the antifungal activity against of *A. flavus*

	MRS broth medium (Control)		*L. rhamnosus*			
pH	6.4		3.6		6.4 (adjusted pH)	
	Inoculated with					
	Spore	Mycelium	Spore	Mycelium	Spore	Mycelium
Fungal growth onset (days)	3	2	-	-	5	5
Fungal spore onset (days)	5	4	-	-	-	-
Final pH	7.49 ± 0.03	9.11± 0.21	3.6	3.6	8.10±0.08	8.90± 0.21
Mycelium dry weight (mg/ ml)	9.20 ± 0.03	11.95± 0.04	ND	ND	8.60± 0.05	8.90± 0.11
Mycelium dry weight reduction %			100	100	6.46	25.50

-: No growth or spore formation ND: Not detected

Table (3) Quantification of organic acids produced by *L. rhamnosus* supernatant

Peak N.	Retention time (min)	Acids	Concentration (mg ml^{-1})	
			C	*L. rhamnosus*
1	2.6765	Formic acid	0.089	1.54 ± 0.04
2	3.8220	Lactic acid	ND	18.54 ± 0.03
3	4.0890	Acetic acid	6.355	7.11 ± 0.06
4	4.3330	Citric acid	2.515	ND
5	5.0580	Unknown	-	ND
6	5.4200	Succinic acid	4.317	7.15 ± 0.03
7	7.7660	Unknown	-	ND
8	8.2120	Propionic acid	0.792	ND
9	10.9710	Unknown	-	ND

C: Control of MRS broth medium ND: Not detected -: Not calculated

III.9 Antifungal activity of *L. rhamnosus* supernatant *in situ* (slice of processed cheese):
The growth and spore onset of *A. flavus*, *A. niger*, *Mucor* sp., *Rhizopus stolonifer*, *Alternaria citri*, *A. niger* ch01 and *A. flavus* ch02 on the control SPC agar medium inoculated by fungal spores Table (4) were 2-3 and 2-5 days, respectively, while when inoculated by mycelia were 2 and 2-4 days, respectively. The growth and spore onset of *A. flavus*, *A. niger*, *A. niger* ch01 and *A. flavus* ch02 on the control processed cheese slices (inoculated by fungal spores) were 2 and 3 days, respectively. *Rhizopus stolonifer* and *Alternaria citri* spores appeared no growth and no sporulation on the processed cheese slices during the incubation at 27 ºC for 14 days. *Mucor* sp. spores appeared no sporulation on the processed cheese slices during the incubation at 27 ºC for 14 days. *L. rhamnosus* supernatant prevented the growth and spore formation of *A. flavus* and *A. flavus* ch02 when cheese was inoculated with spore suspension. *L. rhamnosus* delayed the growth and spore onset of *A. niger* and *A. niger* ch01 by 4 and 5 days, respectively compared to control of processed cheese slices (2 and 3 days, respectively) as shown in (Table 4 and Fig. 3). While, the supernatant prevent the growth and sporulation of *Mucor* sp. compared with control of processed cheese slices (4 and 0 days, respectively). *Rhizopus stolonifer* and *Alternaria citri* had no growth or sporulation on the control of processed cheese slices. The growth and spore onset of *A. flavus*, *A. niger*, *Rhizopus stolonifer*, *A. niger* ch01 and *A. flavus* ch02 were (2 and 2 days), (2 and 3 days), (2 and 3 days), (2 and 3-4 days) and (2 and 2 days), respectively when inoculated on SPC agar medium or control of processed cheese slices. While, no growth or sporulation were detected for *Mucor* sp. and *Alternaria citri* when inoculated on SPC agar medium or processed cheese slices. Supernatant of *L. rhamnosus* delayed the growth and spore onset of *A. flavus*, *A. flavus* ch02, *A. niger*, *A. niger* ch01 and *Rhizopus stolonifer* by 2 and 2-3 days for growth and spore onset, respectively.As mentioned before, the antimicrobial effects are due to the metabolic compounds and their interactions. In addition, the variation of different types of fungi in response to acid stress condition (Gerez *et al*., 2009). In addition, the growth conditions of lactic acid bacteria such as the availability of nutrients, incubation temperature, atmosphere, medium pH and viscosity, could have an effect on the production of antifungal compounds (Fernandez *et al*., 2017). In previous studies, Cosentino *et al*. (2018) found that mixing LAB cultures together on Caciotta cheese succeeded in prevent or delaying some molds. Muhialdin *et al*. (2011) found that LAB supernatant delayed the fungi for 5 to 6 days at 20 and 30 ºC in processed cheese, respectively. Ouiddir *et al*. (2019) founds that some *Lactobacillus* ssp. could slow the fungal growth in sour cream without effecting sensory properties. These results are logical because the most sensitive growth stage to inhibition is conidia germination (Gerez *et al*., 2009). The results suggested that *L. rhamnosus* can be used to prevent the growth of *A. flavus* on the surface of cheese.

Table (4) Antifungal activity of *L. rhamnosus* supernatant on processed cheese slices during incubation at 27 ºC for 14 days

Target fungi	Inoculation by											
	Spores						Mycelium					
	SPC agar medium		Cheese				SPC agar medium		Cheese			
	Control		Control		*L. rhamnosus* supernatant		Control		Control		*L. rhamnosus* supernatant	
	G	S	G	S	G	S	G	S	G	S	G	S
A. flavus	2	2	2	3	-	-	2	2	2	2	4	4
A. niger	2	3	2	3	4	5	2	3	2	3	4	5
Mucor sp.	2	3	4	-	-	-	2	3	-	-	-	-
Rhizopus stolonifer	2	3	-	-	-	-	2	3	2	3	4	5
Alternaria citri	3	5	-	-	-	-	2	4	-	-	-	-
A. niger ch01	2	3	2	3	4	5	2	4	2	3	4	5
A. flavus ch02	2	2	2	3	-	-	2	2	2	2	4	4

Control growth medium: SPC agar G: Growth onset (days) S: Spore onset (days) -: No growth or sporulation until 14 days

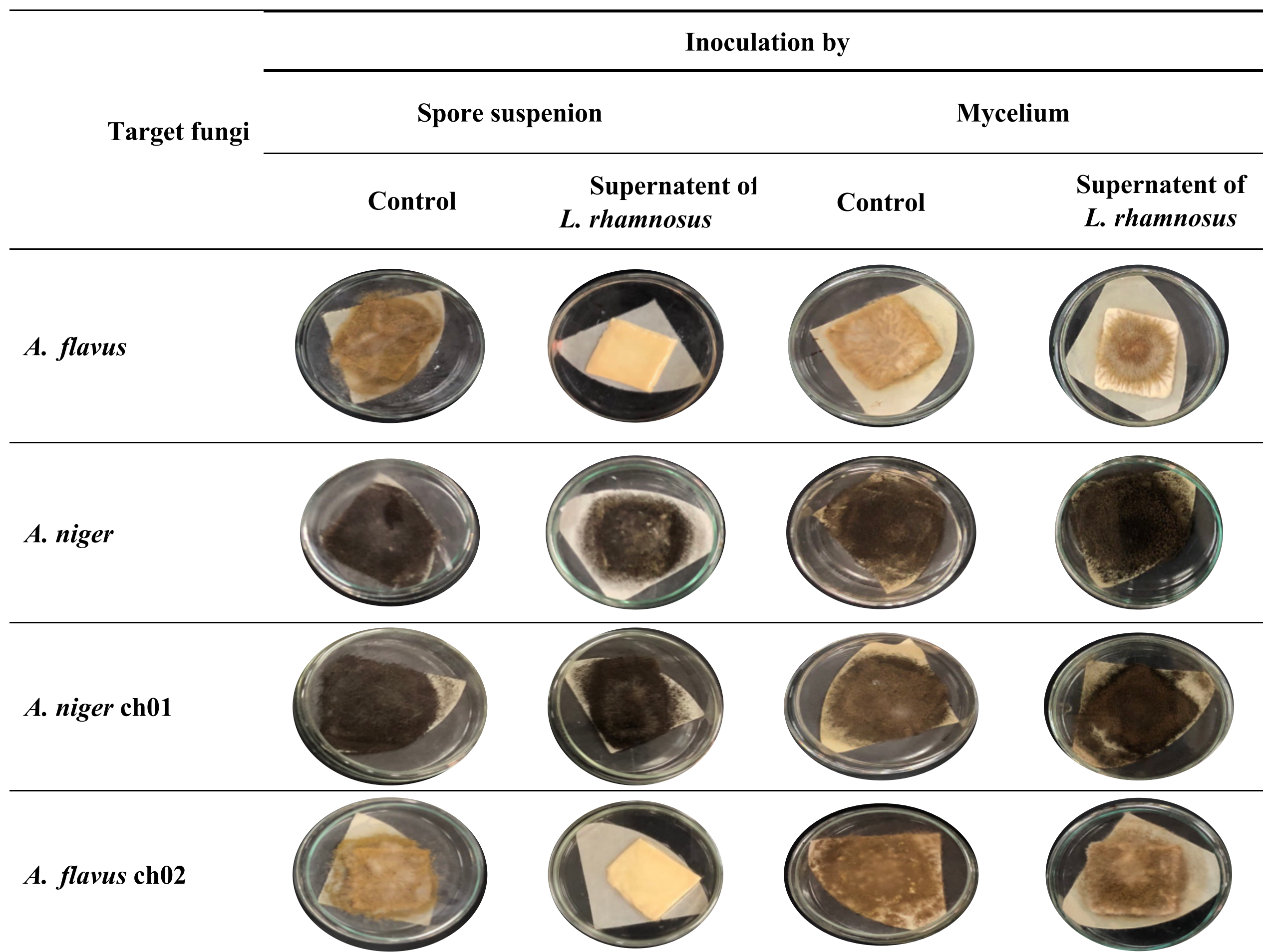

Fig. (3) Antifungal of *L. rhamnosus* against the growth of different spore suspensions and fungal mycelium on cheese slices after incubation 27 ºC for 14 days

CONCLUSION

The obtained results proved the potential impact of some LAB as antifungal strain for use as bio-protective agents against a wide spectrum of molds. However, the antifungal activity can vary according to the contamination way either spores or mycelia. The main organic acids detected in the CFS of *L. rhamnosus* were formic, lactic, acetic, and succinic acids as effective antifungal bio-preservatives.

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النشاط المضاد للفطريات لبكتيريا حمض اللاكتيك في المختبر وفي المادة الغذائية كمادة حافظة حيوية

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تم عزل ٢٠٨ عزلة من منتجات الالبان المختلفة ,أظهر الفحص الأولي أن ٨٠ عزلة منها كانت بكتيريا حامض اللاكتيك. اختبرت هذه العزلات لخصائصها المضادة للفطريات لمنع نمو جراثيم فطر *Apregillus flavus*. دلت النتائج ان ٤ عزلات أظهرت نشاط مضاد لجراثيم فطر *A. flavus*. كما تم عزل ٢ عزلة فطرية من الجبن الراس وتم تعريفهما بـ *A. niger* ch01 و*A. flavus* ch02. تم اختبار قدرة الـ ٤ عزلات من بكتيريا حامض اللاكتيك لمنع نمو جراثيم وهيفات فطريات *A. flavus, A. niger, Mucor sp.,* *Rhizopus stolonifer, Alternaria citri, A. niger* ch01 and *A. flavus* ch02. واظهرت النتائج ان العزلة رقم B50 قد منعت نمو جراثيم جميع الفطريات المختبرة بالأضافة الي قدرتها علي منع او تأخير نمو هيفاتهم. وبناء علي ما سبق تم اختيار العزلة رقم B50 كبكتيريا مضادة للفطريات وتم تعريف هذه العزلة علي انها *Lacticaseibacillus rhamnosus*. عند معادلة pH المزرعة من ٦,٣ الي ٦,٤ والتلقيح بجراثيم أوهيفات فطر *A. flavus* فقد اختفي هذا النشاط ليتضح بأنه يرجع لانخفاض الـ pH. تم تعريف بعض الاحماض العضوية التي تنتجها *L. rhamnosus* بواسطة HPLC و تشمل حمض الفورميك واللاكتيك والخليك والسكسينك. عند اختبار قدرة رائق *L. rhamnosus* المنماه في بيئة MRS السائلة لمنع نموجراثيم وهيفات فطر *A. flavus* و *A. flavus* ch02 من سطح شرائح الجبن المطبوخ اتضح قدرته علي منع نمو جراثيم هذين الفطريين وتاخير نمو هيفاتهما بالأضافة الي تأخير نمو جراثيم وهيفات الفطرين *A. niger* و *A. niger* ch01. مما سبق يتضح امكانية استخدام رائق *L. rhamnosus* لمنع نمو جراثيم فطر *A. flavus* و *A. flavus ch02* من سطح شرائح الجبن المطبوخ.