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Lactiplantibacillus plantarum: A sustainable approach for food additive of fresh-cut strawberry and kiwifruit with bio-control of *Aspergillus spp.*

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Abstract
Aspergillus spp., responsible for grey mold, biological cause of fruit and vegetable spoilage phenomena in post-harvest. Kiwifruit and strawberry are climacteric fruits particularly prone to this mold infestation during storage. Lactic acid bacteria (LAB) are food-grade bacteria that can synthesize several metabolites with antimicrobial activity and are, therefore, suggested as promising and eco-friendly resources for the bio-control of molds on fruits and vegetables. In this work, we propose the screening of a collection of 3 LAB previously isolated from cow milk for their ability to counteract *in vitro* the growth of Botrytis *Aspergillus Niger* ATCC 16888 and *Aspergillus flavus* ATCC 11498. Only 1% of tested LAB strains belonging to *Lactiplantibacillus plantarum* species, exerted strong antagonism against *Aspergillus Niger* ATCC 16888 and *Aspergillus flavus* ATCC 11498. The cell-free supernatants were partially characterized and results clearly indicated that high levels of lactic acid contributed to the antagonistic activity. AV2 cell-free supernatants was investigated as potential bio-control agents in a preliminary *in vivo* assay using freshly cut kiwifruits and strawberry as a food model. The application of cell-free supernatants allowed delay the growth of *Aspergillus Niger* ATCC 16888 and *Aspergillus flavus* ATCC 11498 on artificially contaminated kiwifruits and strawberry until two weeks. The antagonistic activity was greatly affected by the storage temperature (25 ◦C and 4 ◦C) selected for the processed fruits, suggesting the importance to include microbial-based solution in a broader framework of hurdle technologies.

Keywords: *Aspergillus Niger* and *Aspergillus flavus*, post-harvest, kiwifruit and strawberry, lactic acid bacteria, antifungal activity, bio-control, sustainability

Introduction
Freshly cut fruits are getting more and more well-liked because of how convenient they are and how healthy they are. However, the food business faces considerable difficulties due to their short shelf lives and susceptibility to microbial degradation, particularly by fungi like *Aspergillus spp.* (Kadir S, Sidhu G, & Al-Khatib K. (2006) [11]. Using lactic acid bacteria (LAB) for bio preservation. The native micro biota of fresh fruits is called lactic acid bacteria (LAB), and it has been shown that LABs operate as biocontrol agents for a variety of fungi and bacteria in different food products (Batish *et al.*, 1997; Sathe *et al.*, 2007; Linares-Morales *et al.*, 2018; Marín *et al.*, 2019) [17, 21, 22, 23]. It is also essential to remove ethylene from the storage environment. The distinctive strawberry flavor is influenced by sugars, acids, and aroma volatiles, and it depends on the right chemistry of these chemical components. According to Khalil *et al.*, (2021) [24], LAB are rod- or cocci-shaped, gram-positive, nonmotile, nonspore-forming, microaerophilic, and catalase-negative bacteria that ferment carbohydrates to create lactic acid (Akbar *et al.*, 2016) [25]. Bacterial genera such as *Aerococcus, Vagococcus, Tetragenococcus, Oenococcus, Weissella, Pediococcus, Enterococcus, Streptococcus, Leuconostoc*, and *Carnobacterium* make up this diverse group of microorganisms. While sugars and acids are responsible for the fruit's sweetness and tartness, aroma volatiles are what give fresh strawberries their distinctive, delicious flavors. It's crucial to maintain and improve the mature fruit aroma during post-harvest handling because the aroma changes significantly as the fruit ripens after harvest (Forney *et al.*, 2000). The use of synthetic chemical preservatives in traditional fruit preservation techniques raises

issues with food safety, consumer health, and environmental sustainability.

In order to address the problem of *Aspergillus spp*. contamination, this study explores the potential of adopting *Lactiplantibacillus plantarum*, a lactic acid bacterium with probiotic qualities, as an environmentally benign substitute for the bio-preservation of fresh-cut strawberries and kiwifruits.

Table 1: Causative agents in microbiological spoilage of fruits

Causative agents	Spoiled fruits	References
Escherichia coli *Klebsiella sp.*	Pineapple, papaya	Hasan & Zulkahar 2018 [13]
Bacillus sp. *Staphylococcus sp.*	Banana	Hasan & Zulkahar 2018 [13]
Lactobacillus plantarum *Bacillus cereus* *Bacillus subtilis* *Micrococcus luteus* *Pseudomonas aeruginosa* *Staphylococcus aureus* *Proteus vulgaris* *Streptococcus pyogenes* *Erwinia cacticida* *Serratia marcescens*	Apple, watermelon, pineapple, pawpaw, tomato, orange, banana, etc.	Ajayi-Moses *et al.*, 2019 [14]
Aspergillus flavus *Aspergillus Niger* *Aspergillus oryzae* *Aspergillus tubingensis* *Aspergillus foetidus* *Aspergillus awamori* *Aspergillus japonicus* *Aspergillus phoenicis* *Rhizopus stolonifer* *Fusarium oxysporum*	Mango, apple, orange, peach, kiwi, lemon, pokhara (lotus fruit), apricot, tomato, dates, banana, grapes	Al-Hindi *et al.*, 2011 [15]
Aspergillus Niger *Aspergillus fumigatus* *Aspergillus parasiticus* *Aspergillus flavus* *Mucor racemosus* *Mucor piriformis* *Fusarium solani* *Fusarium oxysporum* *Fusarium avenaceum* *Penicillium expansum* *Penicillium digitatum* *Rhizopus oryzae* *Rhizopus stolonifer* *Saccharomyces cerevisiae* *Alternaria alternata* *Kluyveromyces marxianus* *Candida krusei* *Candida tropicalis* *Torulopsis fragaria* *Pichia anomala (Wickerhamomyces anomalus)* *Pichia kluyveri* *Pichia fermentans* *Zygosaccharomyces bailii* *Zygosaccharomyces rouxii* *Geotrichum candidum*	Apple, watermelon, pineapple, pawpaw, tomato, orange, banana, etc.	Ajayi-Moses *et al.*, 2019 [14]

2. Materials and Methods

2.1. Microbial Strains and Growth Conditions: Three LAB strains were isolated from cow milk. Target spoilage strains were *Aspergillus Niger* ATCC 16888 and *Aspergillus flavus* ATCC 11498 in the screening experiment. Lyophilized culture was reconstituted in 0.9% sodium chloride solution, plated on potato dextrose agar (PDA, Hi Media), and incubated at 25 °C for five days. By using a sterile swab to lightly brush the plate surface with saline solution, a fungal spore suspension was created. This suspension was then refrigerated at 4°C for up to 7 days. Placing repeated dilution on PDA plates throughout this time revealed a constant quantity of fungus spores.

2.2. Screening of Antifungal Activity: The antifungal activity of LAB strains against *Aspergillus Niger* ATCC 16888 and *Aspergillus flavus* ATCC 11498 was quickly initially screened using the overlayed technique. According to previously created standard growth curves, 5 µL of late exponential phase cultures (corresponding to roughly 16 h of incubation) were spotted on MRS agar plates and incubated at 30°C for 24 h. Then, 10 mL of Malt Extract (Hi

Media) Soft Agar (0.75% agar) was applied to the plates, and *Aspergillus Niger* ATCC 16888 and *Aspergillus flavus* ATCC 11498 suspensions were inoculated (1:100 v/v) into it. After three days of incubation at 25°C, the halo of inhibition surrounding the spots was used to distinguish the LAB strains and classify them as having no (-), mild (+), or strong (++) inhibition, depending on whether the inhibition zone measured less than 1 mm, 1 to 5 mm, or more than 5 mm. The assays were carried out twice.

2.3. Molecular identification of lab species: The LAB strains showing the best antifungal performance (inhibition halo higher than 10 mm) were identified by sequencing of 16S rRNA. DNA was isolated from the culture provided by the customer. Quality of DNA was evaluated on 1.0% Agarose Gel, a single band of high-molecular weight DNA was observed. Fragment of 16S rRNA gene was amplified by PCR. A single discrete PCR amplicon band was observed when resolved on Agarose. The PCR amplicon was purified by column purification to remove contaminants. DNA sequencing reaction of PCR amplicon was carried out with 27F & 1391R primers using BDT v3.1 Cycle Sequencing Kit on ABI 3500xl Genetic Analyzer. The 16S rRNA sequence was used to carry out BLAST with the database of NCBI GenBank database. Results were submitted for comparison with sequences available at the NCBI database (GenBank) using the standard nucleotide-nucleotide homology search Basic Local Alignment Search Tool. Identification of the isolate AV2 on the basis of 16S rRNA analysis After identifying the genera of the isolated bacteria AV2 by basic characterization procedure described in Bergey's Manual of Systemic Bacteriology, the isolate was further identified up to species level by 16S rRNA sequencing. For this, the bacteria were sent to "Genexplore Diagnostics & Research Centre Pvt. Ltd." Ahmedabad, Gujarat, India.

2.4. Partial Characterization of the antifungal activity of lab cell-free supernatant: The LAB strains showing strong antifungal ability were grown until the late exponential and stationary phase by incubation in MRS at 30°C for 24 and 48 h, respectively. Growth curves were determined by monitoring the optical density at 600 nm (OD600) for 48 h using the plate reader UV-visible spectrophotometer (Agilent technologies, Cary 60). The corresponding cell-free supernatants (identified as AV2) were obtained by centrifugation (8000×g×5 min) and filtration (0.22µm pore size, PVDF syringe Filter). In order to partially characterize the metabolites responsible for the antagonistic activity, an aliquot of the CFSs was neutralized (pH = 7) by adding KOH 1 M. The antifungal activity of CFSs was determined by using the method of radial growth inhibition of hyphae (Wang, H., Yan, *et al.*, 2012) [9] Briefly, PDA plates were supplemented with 20% (v/v) of CFSs, and 10 µL of a freshly-prepared solution containing approximately 1×10^6 spores/mL of *Aspergillus spp.* were spotted at the center of the plate. The control plates were prepared by adding the same concentration of sterile MRS broth. Inhibition percentage was determined by measuring the radial growth of the hyphae after 24, 48, and 72 h of incubation at 24°C. All the assays were performed in triplicate.

2.5. Lactic Acid Quantification: LAB strains were inoculated from stock (1:1000 v/v) in MRS, and aliquots of cultures were collected at 6, 24, 30, and 48 h of growth, centrifuged and filtered as above. Then, the pH was measured with pH meter (Thermo fisher), and the amount of both L-lactic acid and D-lactic acid determined spectrophotometrically in a plate reader (BioTek) by using a specific enzymatic kit (Bio gamma, Rome, Italy) according to manufacturer's instructions. Three different biological and five technical replicates were carried out.

2.6. Assay for fruit decay: Healthy "Hayward" kiwifruts (Actinidia chinensis var. deliciosa A. Chevalier) and strawberry, (Fragaria) were purchased in a local market, washed twice with sterile distilled water and dried. After drying, fruits were cut and divided into similar pieces by using a sterile lancet. For the assay, kiwifruit and strawberry pieces were artificially contaminated or not with *Aspergillus spp*. at a concentration of about 1×10^6 spores/mL (freshly-prepared) by dipping for 30 s in MRS (control), LAB strain. Then, fruits were air-dried under a laminar flow hood. After drying, three pieces of kiwifruits and strawberry for each treatment were stored in Petri dishes at 25°C for 3 days or at 4°C for 14 days. Each treatment was performed in triplicate. The decay development was monitored daily through image acquisition by using a vision computer system equipped with a digital color camera.

2.7. Analysis of sensorial quality: A group of ten trained panelists performed the sensory evaluations of kiwifruits and strawberry at the end of the storage. Before evaluations, panelists were trained in order to recognize and score the quality attributes. Color, mold occurrence, overall acceptance, visual quality, and freshness were evaluated using a hedonic scale from 1 to 5, where 1 = not edible/100% mold presence, to 5 = very fresh/0% mold presence, with 3 fixed as limit of marketability.

3. Results and Discussion

3.1. Screening of the Antifungal Activity of Lactic Acid Bacteria: Lab from cow milk have been widely investigated for their antifungal activity Tulini, *et al.*, (2016) [19] and successfully proposed for increasing the shelf-life of bakery products (Dal Bello, *et al.*, 2007) [20] In this work, we investigated the effectiveness of three LAB strains isolated from cow milk to contrast *Aspergillus spp*., a specific and diffused spoilage microbe of fruits and vegetables. The LAB strains mainly showed only a poor or modest ability to inhibit the fungal target, using the overlayed method. In fact, based on the inhibition halo, it was found that 98% of the tested LAB strains were barely able to inhibit *Aspergillus Niger* ATCC 16888 and *Aspergillus flavus* ATCC 11498 (inhibition halo lower than 3 mm), while only one strains (2%) exerted a strong antagonism, showing an inhibition halo higher than 10 mm. This result agrees with the analysis of the antifungal activity performed by Cheong *et al.*, [10] which screened about three LAB strains, observing that only one isolates (less than 2%) had a strong antagonistic activity against common cheese spoilage molds belonging to the genera *B. cinerea*, *Penicillium*, and *Cladosporium*, suggesting that the ability to deeply counteract the development of specific fungal targets can be a trait not widely diffused among LAB (probably species- and strain-dependent), regardless from the food matrices.

The strains with the best antifungal performances were selected for further *in vitro* characterization and identified

by sequencing the 16S rRNA (Table 2). The molecular analysis revealed that all the strains belonged to *Lactiplantibacillus plantarum* species (formerly *Lactobacillus plantarum*) Zheng, *et al*. 2020 [7]. This evidence confirmed the general antimicrobial potential of *L. plantarum* species Hu CH, Ren LQ, Zhou Y, & Ye BC, (2019) [6]. and the rising evidence of a possible contribution of *lactobacilli*, particularly *L. plantarum*, in the microbial-based bio-control activity against *Aspergillus spp*. on fruits De Simone, *et al*., 2021 [5].

Table 2: The three lactic acid bacteria (LAB) strains with best antifungal activity against *Apergillus Niger* and *aspergillus flavus*

LAB Strains	Source	*Species*
AV1	Cow milk	-
AV2	Cow milk	*Lactiplantibacillus plantarum* AV2
AV3	Cow milk	-

3.2. Anti-*Aspergillus spp*. activity of cell-free supernatants: In order to determine if the antifungal activity was due to direct antagonism or to the production of some metabolites, the CFSs were collected after 24 (CFS24) and 48 (CFS48) hours of incubation, a time corresponding to the late exponential and late stationary phase, respectively (an example is shown in Figure 1). The pH of each CFS was measured, as reported in Table 3.

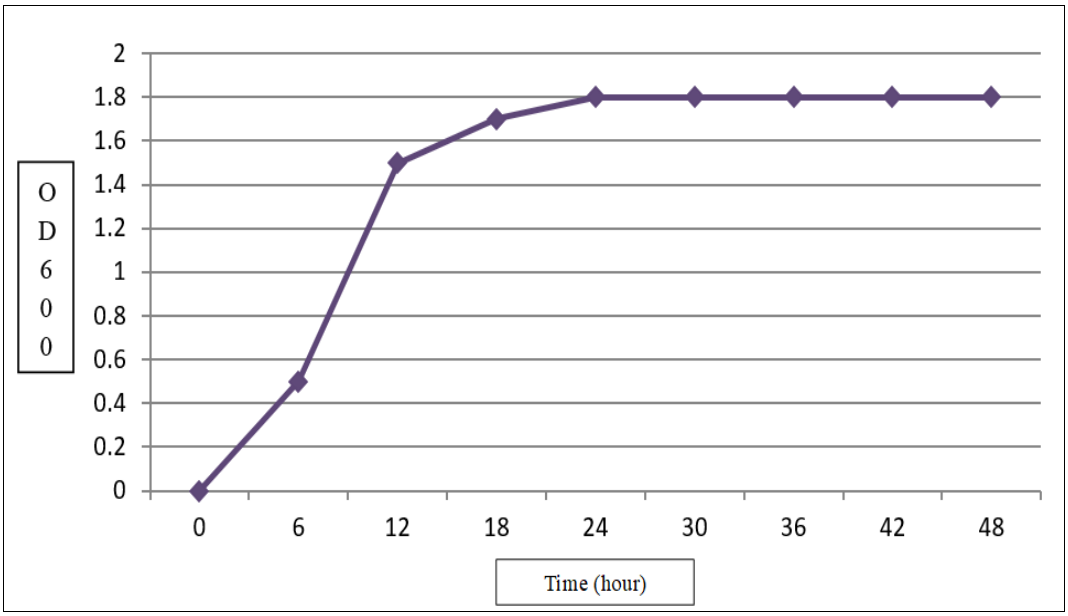

Fig 1: Growth curves of L. plantarum AV2

Table 3: pH of the CFSs obtained after 24 (CFS24) and 48 (CFS48) h of growth in MRS at 37 ◦C.

LAB Strains	pH	
	CFS24	CFS48
AV1	4.11	3.91
AV2	3.82	3.43
AV3	4.22	3.91

Thus, lactic acid production was monitored during 48 h in *L. plantarum* AV2 and selected as the best antagonist strains (Table 4). Since *L. plantarum* can produce both L-lactate and D-lactate, each enantiomer was detected. After 24 h of growth, L-lactic acid production was slightly higher for strain AV2 (about 17 and 14 g/L, respectively).

Table 4: pH and lactic acid (L- and D-enantiomers) production by AV2 strains monitored during 48 h of growth in MRS media.

Time (h)	pH	Lactic Acid (g/L)	L-Lactate (g/L)	D-Lactate (g/L)
6	6.53	0.47 ± 0.06	0.39 ± 0.03	0.11 ± 0.08
24	3.80	16.97 ± 0.57	14.77 ± 0.57	2.17 ± 0.67
30	3.68	20.48 ± 0.78	17.98 ± 0.78	2.58 ± 0.78
48	3.60	27.19 ± 0.31	22.28 ± 0.33	4.93 ± 0.28

3.3. Anti-Aspergillus Activity on Cut Kiwifruits and strawberry: In order to investigate the potential of *L. plantarum* strains as protective treatment to delay the decay during storage of fruit commodities, CFS48 of *L. plantarum* AV2 strains was tested for a preliminary *in vivo* assay by using freshly cut kiwifruit and strawberry as a food model. CFS were applied by dipping, a process usually employed to transfer antimicrobial, antibrowning, or texture preservative compounds to fresh-cut products. A fungicide (e.g., Fenhexamid) classified as a minimal risk to human health and environment for the control of grey mold in pre- and post-harvest was used to compare the efficacy of the proposed approach. Kiwifruits and strawberry were stored for 3 days at 25 ◦C, to mimic a thermal abuse that could encourage the development of the spoiler, and at 4 ◦C for 14 days, simulating a correct management of the cold chain. As expected, when the assay was carried out at room temperature (Figure 2Aand 2B) a fast development of *Aspergillus spp*. was detected on all the artificially contaminated samples, although the fungal growth seems to be delayed in kiwifruits and strawberry treated with CFS48 from AV2 strain and the chemical.

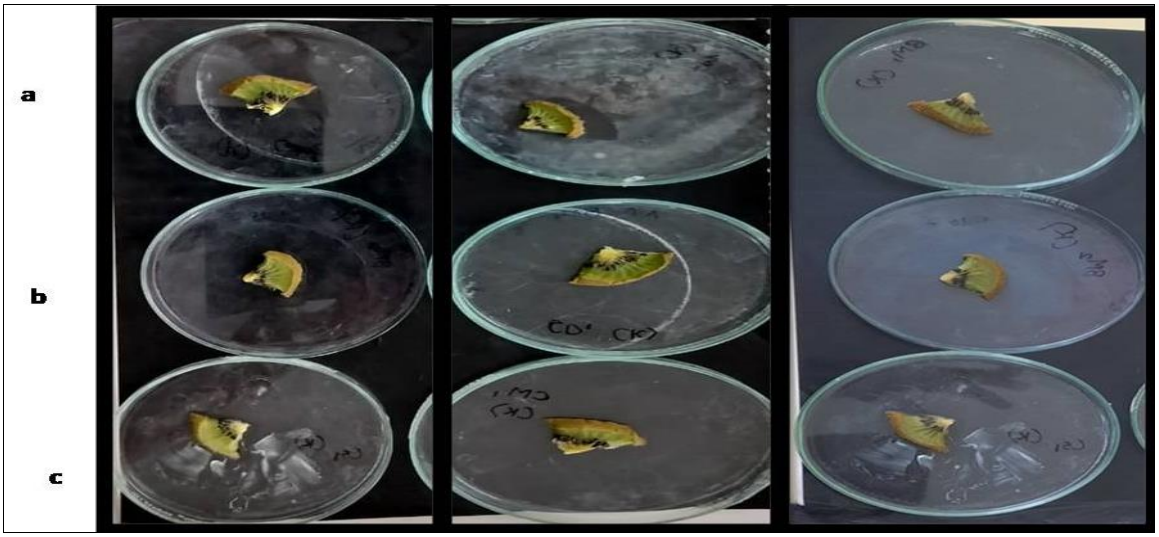

Fig 2A: Image acquisition of kiwifruit pieces non- (a) or artificially contaminated with *Aspergillus spp*_ (b) and treated with Fenhexamid (c), CF S48 of *L. plantarum* AV2 and stored for 3 days at 25 ºC _

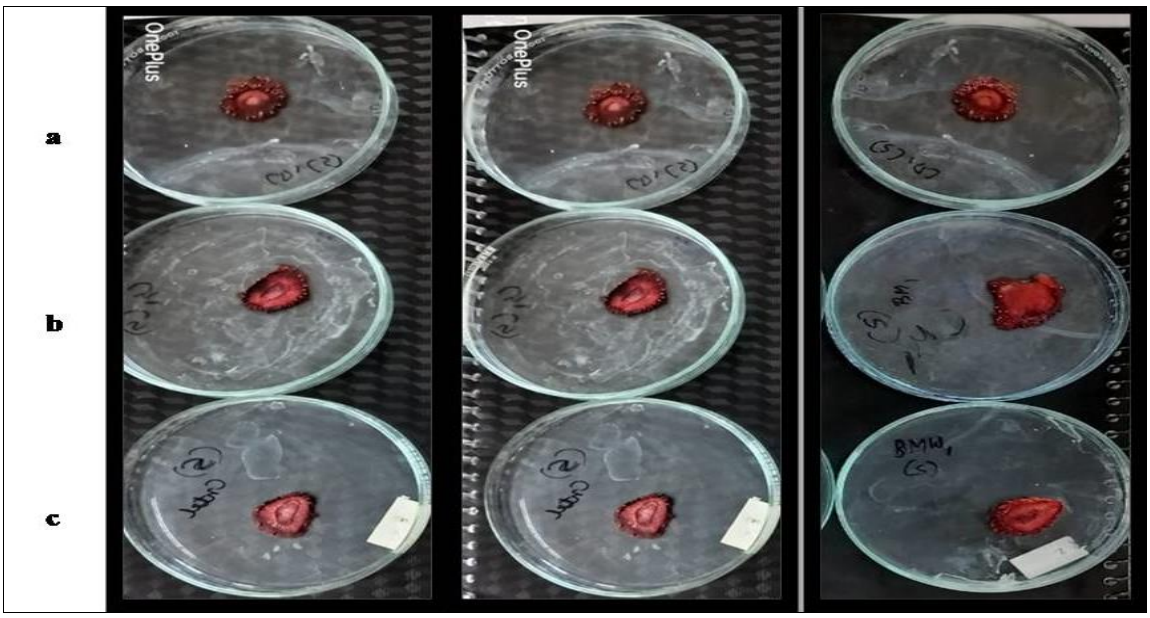

Fig 2B: Image acquisition of Strawberry pieces non- (a) or artificially contaminated with *Aspergillus spp*_ (b) and treated with Fenhexamid (c), CF S48 of *L. plantarum* AV2 and stored for 3 days at 25 ºC _

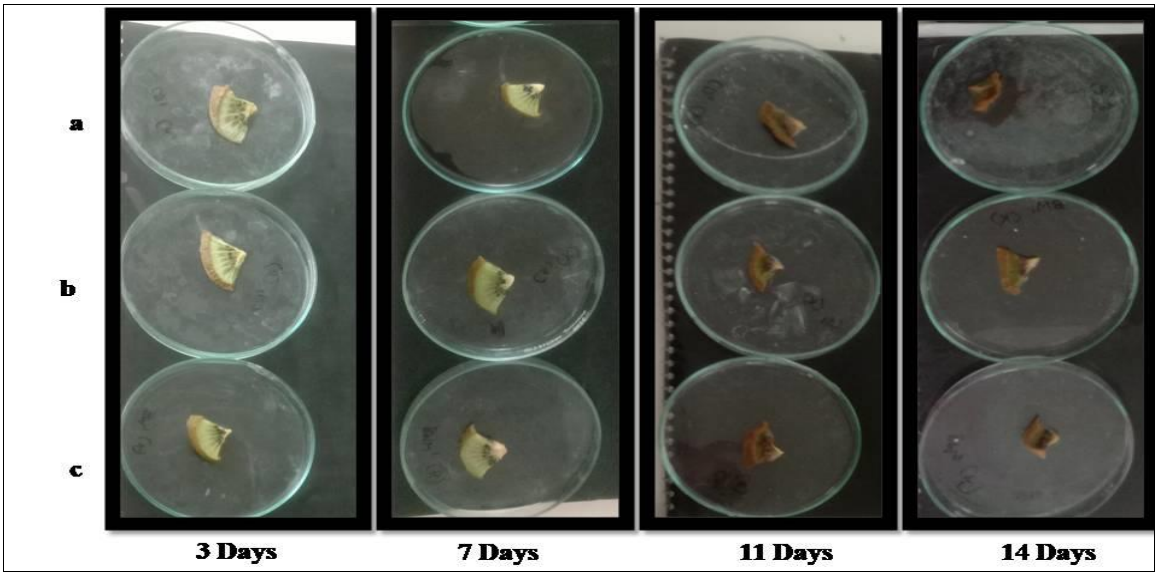

Fig 3C: Image acquisition of Kiwifruit pieces non- (a) or artificially contaminated with *Aspergillus spp*_ (b) and treated with Fenhexamid (c), CF S48 of *L. plantarum* AV2 and stored for 14 days at 4 ºC

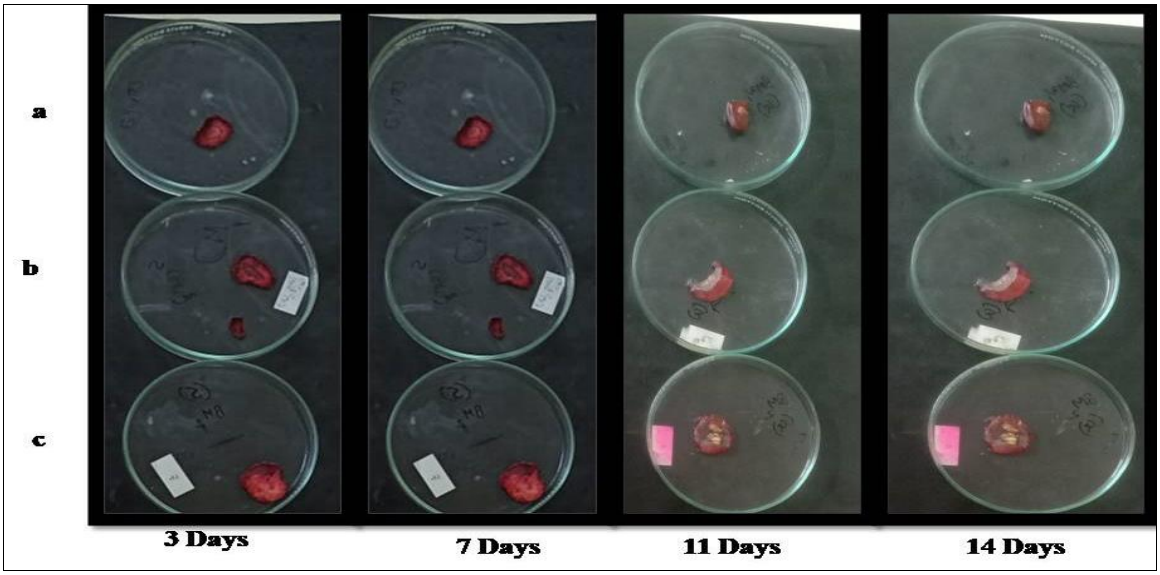

Fig 2B: Image acquisition of Strawberry pieces non- (a) or artificially contaminated with *Aspergillus spp*_ (b) and treated with Fenhexamid (c), CF S48 of *L. plantarum* AV2 and stored for 14 days at 4 ºC _

Under cold storage conditions (Figure 4A& 4B), *Aspergillus spp.* began to develop only after the seventh day in non-treated contaminated samples, covering the whole fruit's surface on the tenth day. In contrast, molds occurrence was only minimally detectable after two weeks in kiwifruits and strawberry submitted to CFS48 treatments. No fungal growth was disclosed in control and chemically-treated samples. In particular, kiwifruits and strawberry stored at 4 ◦C were subjected to several modifications, such as color changes, loss of firmness, dehydration of the cut surfaces, probably associated with alterations in nutritional and organoleptic quality, started to occur after 10 days of preservation, regardless of contamination with *Aspergillus spp.* (Figures 3A and 3B). These changes could be induced by biochemical reactions associated with cell senescence, accelerated by unit operations, such as cutting and washing. Figure 3A and 3B show changes in sensory parameters after 10 days and at the end of storage time in cold-stored kiwifruits and strawberry. As expected, it was observed that artificial contamination of kiwifruits and strawberry pieces with *Aspergillus spp*. greatly affected the product's quality. Control and treated with Fenhexamid samples showed the best performance during storage, being still marketable after two weeks. At the same time, kiwifruits treated with CFS48 from *L. plantarum* AV2 showed better behaviors than other strain reaching not acceptable overall quality anyhow. Interestingly, after 10 days of cold storage, the kiwifruit and strawberry pieces dipped in CFS48 of AV2 was considered to be of sufficient quality for marketing, as no significant differences were found with the control fruits.

Therefore, our results suggest that, despite the LAB strains analyzed might not be used for applications in which a complete inhibition of *Aspergillus spp*. is required, they could still be valuable in the design of protective microbial-based solutions to delay its growth, extending shelf life and improving fruit marketability. In particular, it was further confirmed the broad antifungal activity of strain AV2 in different food matrices (Arena MP, *et al.*, 2019) [2], indicating potential applications also in the biocontrol of fruit products. Moreover, in this study, conditions encouraging fungal contamination, including a high level of spores and packaging in passive atmosphere, have been evaluated, suggesting potential greater bio-control effectiveness than what was observed. Accordingly, it has been reported that that the antimicrobial effectiveness of live bacteria on fresh-cut fruits was positively correlated with antagonist concentration (Abadias M, *et al.*, 2014) [3] However, the addition of viable bacteria could drive detrimental fermentations, leading to off-flavors' production impacting the overall quality of the fruits Russo P, *et al.*, 2015) [4].

4. Conclusion

Due to their possible capacity to create mycotoxins, filamentous fungus is the cause of serious food deterioration and safety issues. LAB has been the subject of much research recently due to their potential to inhibit fungus growth, and this makes them a viable option for the bio-conservation of fruits and vegetables. In particular, increasing shelf life without the addition of chemical additives is one of the main challenges for the sector. In this work, we selected *Lactiplantibacillus plantarum* strains from a large cohort of LAB based on their ability to contrast the growth of *Aspergillus spp*. likely due to the production of organic acids. To increase safety and shelf life without compromising the overall quality of the product, more research should be conducted on cutting-edge technologies for delivering antifungal metabolites of microbial origin and their combination with physical therapies.

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