Antifungal Activity of a Carboxy Methyl Cellulose-Aloe vera-based Edible Coating as a Carrier of Living Cells in Pistachio Nut

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Information

Article Type:
Original Article

Article History:

Received: 13.07.2022
Accepted: 02.09.2022

Doi: 10.22123/PHJ.2023.375582.1143

Keywords:
Fresh pistachio
Aloe vera, *Lactobacillus plantarum*, *Bacillus subtilis*, *Aspergillus flavus*

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Abstract

Background: The incorporation of live microbial cells in edible films has been considered a suitable biocontrol approach to increase the shelf life of food products.

Materials and Methods: The current study investigated the possibility of developing an Aloe vera gel-based edible film containing *Bacillus subtilis* B5 and *Lactobacillus plantarum* DSMZ 20174 and their inhibitory effect against the growth of *Aspergillus flavus* in fresh pistachios during 30 days of storage.

Results: The results indicated that the sporulation dramatically decreased with an increase in the concentration of *Lactobacillus plantarum* to 10^7 CFU/ml and that any sporogenesis was detected at 10^8 CFU/ml. Significant differences were observed in the inhibitory effect of *Bacillus subtilis* (90%) against *Aspergillus flavus* compared to *Lactobacillus plantarum* (60%). By observing the opacity of the wells, any trace of sporulation was noticed at 10^2 to 10^8 CFU/ml *Bacillus subtilis* concentrations. By reducing the inoculum volume of *Bacillus subtilis* cells to 10^1, the inhibition percentage was reduced to 76%, continuing to prove the antifungal effectiveness of this bacteria against the *Aspergillus flavus* growth even in a decreased inoculated population.

The edible film containing *Bacillus subtilis* and *Lactobacillus plantarum* demonstrated an inhibitory effect on the growth of *Aspergillus flavus* in fresh pistachios, with *Bacillus subtilis* having the most significant impact.

Conclusion: The application of the Aloe vera-based coating bearing live cells could be considered an appropriate, safe, and economical approach to prevent *Aspergillus flavus* growth and aflatoxin production in fresh pistachios.

►Please cite this article as follows:
Ebrahimzadeh Mousavi Z, Ghasemi M, Mousavi M, Mirzadi Gohari A, Niknam R. Antifungal Activity of a Carboxy Methyl Cellulose-Aloe vera-based Edible Coating as a Carrier of Living Cells in Pistachio Nut. Pistachio and Health Journal. 2022;5(3):25-36.

1. Introduction

Pistachio has been considered part of the daily human diet since prehistoric times; it is well-known for its high nutritional value, pleasant taste, health benefits, and significant economic impact [1-3]. Pistachio is mainly cultivated in the Middle East, Central Asia, and the Mediterranean region. Generally, it has low water activity, significantly reducing the risk of spoilage by microorganisms. However, some fungi could even grow on low-moisture foods, including nuts [4]. Pistachio is among the most susceptible nut to fungal infection. This fungal infection is mostly caused by *Aspergillus niger* and *Aspergillus flavus*, which could provoke aflatoxin contamination in this nut [4, 5]. Various studies have confirmed the contamination of pistachio nuts with aflatoxins at different cultivation steps, as well as post-harvest, drying, transport, storage, and processing [3, 4, 6].

Mycotoxins are fungal secondary metabolites with a low molecular weight that have toxic effects on animals and humans. The main adverse effects include carcinogenicity, mutagenicity, teratogenicity, cytotoxicity, neurotoxicity, nephrotoxicity, immunosuppression, and estrogenic effects [3, 7, 8].

Due to the negative effect of agrochemical consumption on the environment and human health, the need for other eco-friendly, low-cost approaches for controlling fungal contamination in foods is increasing [9]. One of the main non-destructive food preservation methods facilitating extended shelf life and increasing food safety in food products is edible coating [10, 11]. The edible coating is a thin layer of natural polymers applied to food surfaces using different techniques [10, 12] . These coatings are appropriate carriers for bioactive substances such as antioxidants, antimicrobials, and nutrients. Being combined with beneficial microorganisms, the edible coating could promote the biological preservation of food products, which results in extending shelf life and preventing food quality and sensory properties from deteriorating [13-17].

It has been reported that some bacterial species, such as Lactic Acid Bacteria (LAB), can suppress the growth of aflatoxin-producing fungi; they can degrade aflatoxins and convert them to a non-toxic compound. This ability is attributed to the enzymes and metabolites such as acids, carbon dioxide, hydrogen peroxide, phenyl lactic acid, and bioactive low molecular weight peptides excreted by this group of bacteria [15, 16, 18, 19]. On the other hand, several studies have revealed that some probiotic LAB could inactivate aflatoxin AFB1 through adsorption [20-22].

In addition, some other bacterial species, such as *Bacillus species*, degrade aflatoxins by producing extracellular enzymes and antifungal peptides [23-25].

Gajbhiye *et al.* [26] reported that *Bacillus subtilis* AFB22 could prevent pomegranate spoilage caused by *A. clavatus*, *A. flavus*, *R. stolonifer*, and *F. graminearum* through the production of antifungal metabolites. *Bacilllus subtilis* V26 could inhibit the growth of *Botrytis Cinerea* in tomatoes [27], and the novel antifungal peptides discovered in *Bacillus subtilis B25* inhibits the growth of *Fusarium oxysporum* in bananas [28].

Therefore, according to the previous studies on edible coatings and their capacity to carry antimicrobial substances to prevent fungal growth and aflatoxin, the present study aimed to investigate the use of Carboxy Methyl Cellulose Aloe vera-based coating bearing *lactobacillus*

plantarum and *Bacillus subtilis B5* to prevent *A.flavus* growth in pistachio nut during storage.

2. Material and Methods

Lactobacillus plantarum DSMZ 20174 and *Aspergillus flavus* B16 were provided in agar plate by the microbiology laboratory at the Department of Food Science and Engineering, University of Tehran. *Bacillus subtilis* B5 was previously isolated from Iranian pistachio by Farzaneh *et al.* [29]. Carboxymethyl cellulose (Qlab, China), Gelatin type B (Merck, Germany), Citric acid (Merck, Germany), and Glycerol (Merck, Germany) (as plasticizer) were provided. In addition, Tween 80 (Merck, Germany) was used to prepare the fungal suspension, and sodium hypochlorite was applied to sterilize pistachios [30]. Fresh pistachios were purchased from the local market and kept at 4°C for further experiments.

2.1. Extraction of Aloe vera Gel

Aloe vera leaves were purchased from the greenhouse study center located in the Faculty of Agriculture & Natural Resources of the University of Tehran, Iran. At the initial step, fresh and mature aloe vera leaves were rinsed with tap water and peeled off. The gel was separated and transferred into a blender. Then, the blended gel was filtered to remove the fibers. Subsequently, the filtered gel was pasteurized at 70°C for 45 minutes and cooled down to ambient temperature. Thereafter, the pH of the gel was adjusted to 3.2 using Citric acid (Merck, Germany). Finally, the gel was stored in a foil-covered glass jar and kept refrigerated to prevent oxidation till further evaluation.

2.2. Preparation of Bacterial Cells

Lactobacillus plantarum was activated by streaking on MRS agar medium (Merck, Germany) and incubating at 37°C for 48 hours. *Bacillus subtilis* UTBSP1 was activated in nutrient agar medium (Merck, Germany) in an incubator at 30°C for 72 hours. The activated cells were then suspended in a broth medium and were grown overnight at 30°C. Afterward, 1 ml of the overnight cultures prepared were centrifuged under sterile conditions for 10 minutes at 6000 rpm. Then, the supernatant was discarded, and the cells were washed twice with a sterilized saline solution. Eventually, the washed cells were transferred to the coating solution.

2.3. Preparation of Fungal Suspension

A. flavus B16 was grown on PDA (Potatoe Dextose Agar, Sigma, Germany) at 30 °C for 7 days to sporulate. A sterilized surgical blade was used for scraping obtained spores from the culture surface; then, they were suspended in sterilized distilled water containing Tween 80 (0.1% v/v). The suspension was filtered through a sterile Whatman filter paper (Merck, Germany) under sterile conditions to remove mold mycelia. Eventually, the number of spores in the filtered suspension was adjusted to 10^4 spore/ml using a hemocytometer (Merck, Germany).

2.4. Preparation of Edible Coating Solution

A 1% w/v solution containing Carboxymethyl Cellulose and Gelatine in equal proportions was produced by gradually dissolving 0.5 g of Carboxymethyl Cellulose powder in 100 ml of Aloe vera gel at 70 ° C and 500 rpm for 40 minutes. Then, 0.5 g of Gelatine powder was added to the mixed solution and stirred gently for 30 minutes. Next, Glycerol (50% of the dry matter) was added as a plasticizer and heated for 20 minutes. Finally, the prepared solution was heated at 80°C for 10 minutes to eliminate microbial contamination.

The solution was then cooled to 30-37°C, and the already prepared microbial suspensions were added separately to the coating solution and stirred gently for 10 minutes. A coating solution without any added cells was considered a control sample

2.5. Physical and Mechanical Properties of the Aloe vera-based Coating

2.5.1. Preparation of Film

In order to investigate the physical properties of the coating, the film was first prepared based on the coating formulation. The prepared solution was first sonicated for 10 minutes at a power of 100W to ensure complete homogenization. Then, 1 ml of the prepared microbial suspension was added to 100 ml of the film solution and stirred gently for 10 minutes. Next, 20 ml of the obtained solution was poured into the plate and dried for 36 hours at 38°C in an oven. The dried films were kept in sealed plastic bags till further investigation. Films without added live cells were considered control samples.

2.5.2. Physical Properties

2.5.2.1. Opacity

The opacity of the films was measured according to Soukoulis *et al.* [31]. Initially, the spectrophotometer (Bio Quest, England) was calibrated with an empty cuvette cell. Then, the films were cut into rectangular pieces and placed perpendicular to the cuvette in the apparatus. The absorbance of the film was recorded using a spectrophotometer at 600 nm. The opacity of the films was obtained using the following equation:

$$\text{Opacity} = \frac{A600}{\text{thickness}} \quad \text{Eq.1}$$

The A_{600} indicates the absorption of the film sample at 600 nm, and the thickness is the thickness of the film sample in mm.

2.5.2.2. Thickness

The thickness of the prepared films was measured using a micrometer with an accuracy of 0.01 mm as the average of eight various points, and the mean of the obtained data was introduced as the film thickness.

2.5.2.3 Water Solubility

The water solubility **(SW)**of the film samples was investigated using Soukoulis [31] method with slight modification. The desiccated film samples by P2 O5 for 7 days were weighted (W_0) and subsequently immersed in a beaker containing 50 ml of distilled water and gently stirred at 25°C for 24 hours. Thereafter, unresolved SEFs were dried to a constant weight (W_1) at 60 °C. The water solubility was calculated according to the following equation:

$$\text{Water solubility (\%)} = \frac{\text{Initial dried weight of the film} - \text{Dried film weight after immersion}}{\text{The initial dried weight of the film}} \times 100 \quad \text{Eq.2}$$

2.5.3. Mechanical Properties

Tensile Strength and Elongation at Break

The tensile strength (TS) was evaluated using a tensile analyzer (Model?). Initially, the films were cut (with dimensions of 8×1 cm) and conditioned in a desiccator containing a solution of saturated Magnesium Nitrate with a relative humidity of 50% at 25°C for 48 hours. The distance between the jaws of the mechanical-tensile analyzer was 40 mm, and their movement speed was 50 mm per minute; three iterations were performed for each film sample.

The tensile strength and elongation at break (EB) parameters were obtained using, respectively, the following equations:

$$TS = \frac{F_{max}}{A_{min}} \quad \text{Eq.3}$$

Where F_{max} is the maximum tensile strength, and A_{min} is the minimum film surface.

$$\%EB = (\frac{L_{max}}{L_0}) \times 100 \quad \text{Eq.4}$$

Where L_{max} is the maximum elongation at break, and L_0 is the initial film length.

2.6. Pistachio Coating with the Prepared Coating Solution

Initially, pistachios (Ahmad-Aghaei cultivar) were purchased from a local market in the city of Rafsanjan, Iran. Pistachio samples were immersed in 4% sodium hypochlorite solution (Scharlau, Spain) under sterile conditions for 2 minutes to reduce the initial microbial load and then washed in sterile distilled water. After that, fresh pistachios were immersed in the prepared coating solution for 2 minutes. The excess solution was drained using a strainer, and the samples were subsequently dried at 25°C under a laminar flow cabinet. In order to artificially contaminate pistachio samples, sterile swabs were used to inoculate fungal spores with a concentration of 10^4 CFU/ml on pistachio surfaces. Then, the coated samples were stored in a sterile plate at 4°C and 25°C for 30 days in the dark.

2.7. Evaluation of Inhibitory Effect of the Coatings on *Aspergillus flavus* Growth

The inhibitory activity of the selected bacteria strains against *Aspergillus flavus* was evaluated using the microdilution method in a 96-well plate. Firstly, 100 µl of double-strength PDB medium was poured into each well. Next, 100 µl of *Aspergillus flavus* spore suspension at a concentration of 10^4 CFU/ml was added. Then, 100 µl of the control coating solution was poured into the first row. In the next two rows, 100 µl of coating suspensions containing 10^8 to 10^1 CFU/ml *L. plantarum* and *Bacillus subtilis* were added to the wells, respectively. The microplate was placed in an incubator at 25°C for 72 hours, and the turbidity of the wells as an indicator of fungal growth was investigated. The inhibitory effect of the three different coatings was calculated based on the following equation (Kadaikunnan, Rejiniemon, Khaled, Alharbi, & Mothana, 2015).

$$\text{Inhibitory effect (\%)} \frac{\text{the opacity of the control sample}}{\text{the opacity of the control sample} - \text{opacity of the experiment case}} \times 100 \quad \text{Eq.5}$$

2.8. Evaluation of *Aspergillus* Growth in Pistachios during Storage for 30 Days

Different pistachio samples were prepared in the following 4 different groups and evaluated every week for 30 days at 4°C and 25°C to determine the antifungal effects of the coating against *Aspergillus flavus* (mycelium growth and sporogenesis):

1- Uncoated pistachios (the control group)

2- Pistachios coated with a coating solution (1% carboxymethyl cellulose and gelatine)

3- Pistachios coated with a coating solution containing *Lactobacillus plantarum*

4- Pistachios coated with a coating solution containing *Bacillus subtilis*

2.9. Evaluation of Cell Viability of Bacteria in the Coating

Coating solutions containing 10^8 CFU/ml of the selected bacteria were prepared and kept at 4°C for 30 days, and live cells were counted at 7-day intervals. The pour plate method was used to count *Lactobacillus plantarum*. In order to count the number of live cells in the coating, 1 ml of the prepared microbial coating suspension was transferred to a sterile plate, and appropriate sterile MRS agar was poured into the plate; the colonies were counted after 48 hours of incubation at 37°C. The spread culture method was used to enumerate *Bacillus subtilis* colonies. 0.1 ml of the microbial suspension was poured into the nutrient agar medium, and the number of colonies per ml was obtained after 24 hours of incubation at 30°C; the results were expressed as CFU/ml.

2.10. Statistical Analysis

Statistical analyses were performed by the Minitab software (version 16.2, USA). In order to determine the significant effects of different coatings on *Aspergillus flavus* growth, the experimental data were subjected to a one-way analysis of variance (ANOVA). The significance level was $p < 0.05$.

3. Results and Discussion

3.1. Physical Properties of the Aloe vera-based Film

Table 1 presents the physical properties of different films based on CMC-Aloe vera. The data demonstrated that adding live cells significantly reduced the transparency and increased the opacity of the edible films compared to the control film (without live cells). According to statistical analysis, the control film and the film containing *Lactobacillus plantarum* had the lowest and highest opacity, respectively. According to previous studies, this phenomenon is the result of the light scattering effect of live cells embedded in the film, affecting light transmission through the film [32-34].

The incorporation of microbial cells into the film matrix significantly ($P<0.05$) increased the film thickness. According to previous similar studies, the increase in soluble solids in the film could increase the film thickness [31, 35, 36]. The original water solubility of the CMC-Aloe vera film was 55%. By adding microbial cells, the water solubility increased to about 57% in *L.plantarum*. WS is characterized by chemical structure and defines the material tolerance or resistance to water [37]. It is supposed that live cells could result in an increase in noticeable micropores or interspaces in the film microstructure, leading to weaker hydrogen bonding inside the film [38].

By adding bacterial cells to the films, their tensile strength was significantly reduced from 1.25 Mpa in the control sample to 0.70 and 0.45 Mps in samples containing *B.subtilis* and *L.plantarum*, respectively. This result authenticated the data reported by Kanmani *et al.* [39], where adding bacterial cells to pure pullulan films reduced the tensile strength. Sánchez-González *et al.* [40] reported that the addition of Lactic Acid Bacteria in Methylcellulose edible films reduced tensile strength. In probiotic films, the cohesion of the polymer chains is broken by the probiotic cells, which results in a significant reduction in tensile strength, elongation at the breakpoint, and cohesion of the structure in the samples compared to the controls [33].

The original length elongation to the breakpoint (75%) showed a significant reduction ($P<0.05$) by embedding the *L.plantarum* (44%)

and *B.subtilis* (61%) cells in the film matrix. According to the results, the films containing bacterial cells exhibited less flexibility due to decreased length elongation at the breakpoint, *subtilis*.

3.2. Inhibitory Effect of CMC-Aloe vera Coatings against *Aspergillus flavus Growth*

Evaluation of the inhibitory effect of CMC-Aloe vera-based coating using a microdilution approach revealed that the control film exhibited an inhibitory effect of 20%, possibly related to the antibacterial, antiviral, and antifungal activities of Aloe vera plant extract [41, 42] (Fig. 1) Lengai *et al.* [43]

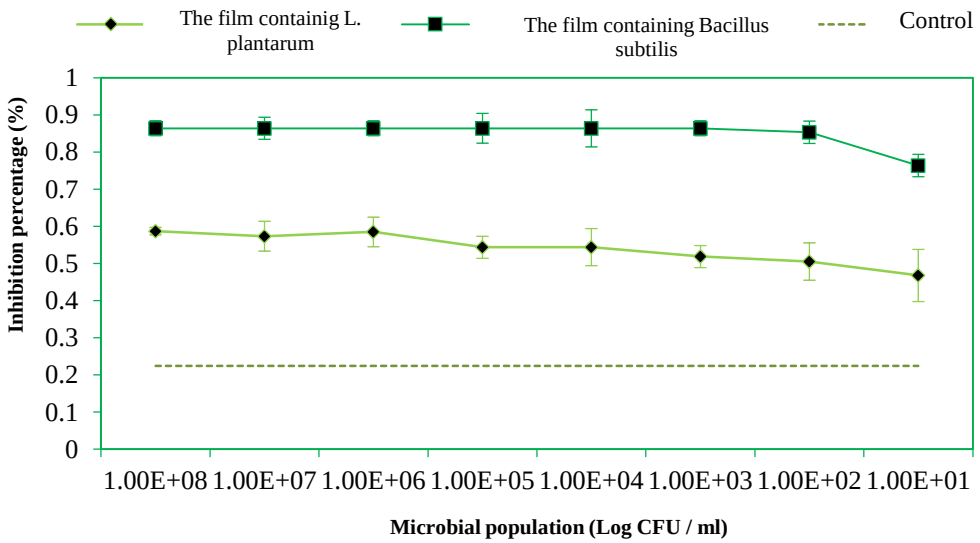

Fig 1. Determination of the antifungal activity of microorganisms (*L. plantarum and B. subtilis*) exploited in Aloe vera-based coating

reported that the antimicrobial activity of A. vera is assigned to the phytochemicals, which denature microbe proteins, thus disrupting their functionality. This natural plant extract can reduce mycelium germination and growth and fungi spore persistence [44]. Radi et al. (2017) confirmed the antibacterial and antifungal effect of gelatin-based edible coatings incorporated with Aloe vera and black/green tea extracts on fresh orange cut [45]. The fungal growth observation in the coating solution containing microbial cells showed the synergistic effect of Aloe vera extract and microbial cells against *A.flavus* growth and mycelium development.

Moreover, by increasing the incorporated cell population in the coating solution from 10^1 to 10^8 CFU/ml, the antifungal effect of the coating was significantly elevated ($P<0.05$). The observation revealed that with the increase in cell concentration of *Lactobacillus plantarum to* 10^7 CFU/ml, the sporulation was significantly decreased (60% inhibitory effect) and fully inhibited at a cell concentration of 10^8 CFU/ml. The antifungal activity of *L. plantarum* is related to the ability of this microorganism to produce metabolites, including hydrogen peroxide, organic acids, cyclic dipeptides, fatty acids, and phenyl lactic acid [46, 47]. Surprisingly, a higher

inhibitory effect against *A.flavus* (90%) was observed in the coating solution containing *B.subtilis*. Any sporulation was detected within the range of 10^2 to 10^8 CFU/ml *B.subtilis*. With the decrease in the inoculum of *B.subtilis* cells to 10^1 CFU/ml, the inhibition percentage reduced to 76%. This high inhibitory effect of *B.subtilis*, even at low cell density, confirmed the effectiveness of this species against *A.flavus* growth and its sporulation. Several pieces of research confirmed the antifungal effect of *B. subtilis* (Mardanova et al., 2016; Reddy, Reddy, & Muralidharan, 2009; YOUCEF-ALI et al., 2014; Zhang, Hu, Wang, Cheng, & Shi, 2007). With the evaluation of the degradation of aflatoxin B_1 by *Bacillus subtilis* UTBSP1 isolated from Iranian pistachios, the researchers reported that the destructive activity of *Bacillus subtilis* is probably due to the extracellular nature of the enzyme produced in the environment that could remove aflatoxin B_1 (Farzaneh et al., 2012).

3.3. Evaluation of Observations of Pistachios Stored at 4°C for 30 Days

The observations showed that the prepared coating solutions, both microbial and non-microbial, could prevent the growth of *Aspergillus flavus* in all samples artificially contaminated with 10^4 CFU/ml spore concentration (Fig. 2).The only difference between the samples containing the microbial and non-microbial coatings was in the appearance and quality of the pistachios. The skin of pistachios coated with non-microbial solution lost moisture and slightly wrinkled after being stored for 30 days. Also, brown spots were observed on the skin of pistachios coated with a non-microbial solution. On the other hand, the pistachio samples coated with microbial solution had better quality, and they remained fresh after 30 days of being stored at 4°C.

Fig 2. *Aspergillus flavus* growth on artificially contaminated pistachios at 4°C: A) control, B) simple coating, C) coating with *Lactobacillus plantarum*, and D) coating with *Bacillus subtilis*

3.4. Evaluation of the Observations of Pistachios Stored at 25°C for 30 Days

The pistachio samples were artificially contaminated with a concentration of 10^4 CFU/ml of spore suspension and kept at 25°C for 30 days to determine the effect of coatings on mold growth. The observations (Fig. 3) and the results of fungal spore counts (Fig.4) showed that in the pistachios without coating, mold growth occurred significantly during 30 days of storage at 25°C. Those with the coating (without live cells) were contaminated at 25°C after 7 days of storage in a closed plate and produced spores. In these samples, the spores count in each pistachio was 2.8×10^6. The sporogenesis process in the coated samples was much slower than in

the control sample; however, at the end of day 30, no apparent difference in the spores counted was observed between coated and control samples. This can be related to the reduction in the exchange of respiratory gases through the coatings. Molds are strictly aerobic and sensitive to the decrease in oxygen. Therefore, the formulated coating alone can enhance the microbial safety of the pistachios. However, adding antimicrobial agents seems necessary to prevent mold growth for a long period of storage time.

In the pistachios coated with microbial coating solutions, the fungal growth was significantly reduced compared with uncoated pistachios and those coated without live cells. Thus, the value of spores counted in the samples with microbial coating was 1.5 Log less than in other samples. Among the microbial coatings, those containing *Bacillus subtilis* had the lowest count of spores among all treatments (10^5 CFU/ml); this is in line with the observations of samples in Fig. 3. The pistachio skin color coated with *Lactobacillus plantarum* completely changed after 14 days, and the fungal mycelium started to grow; in pistachio coated with *Bacillus subtilis* only, the pistachio skin was slightly discolored. At the end of 30 days, the pistachios coated with *Bacillus subtilis* were less contaminated with mold compared with other samples.

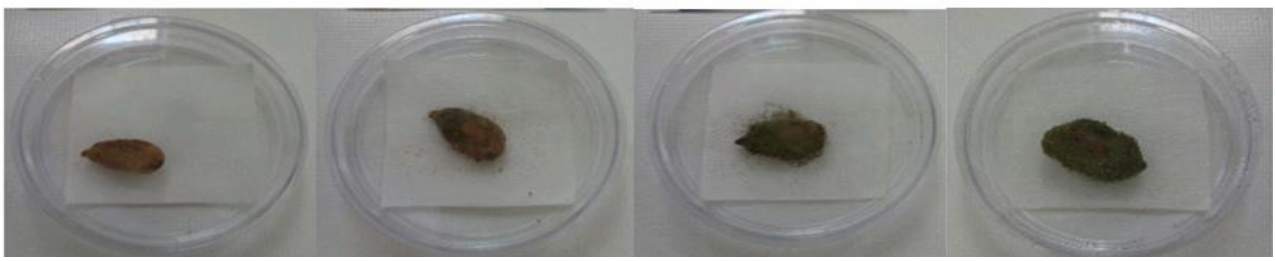

Fig 3. *Aspergillus flavus* growth on infected pistachios at 25°C: A) control, B) simple coating, C) coating with *Lactobacillus plantarum*, and D) coating with *Bacillus subtilis*

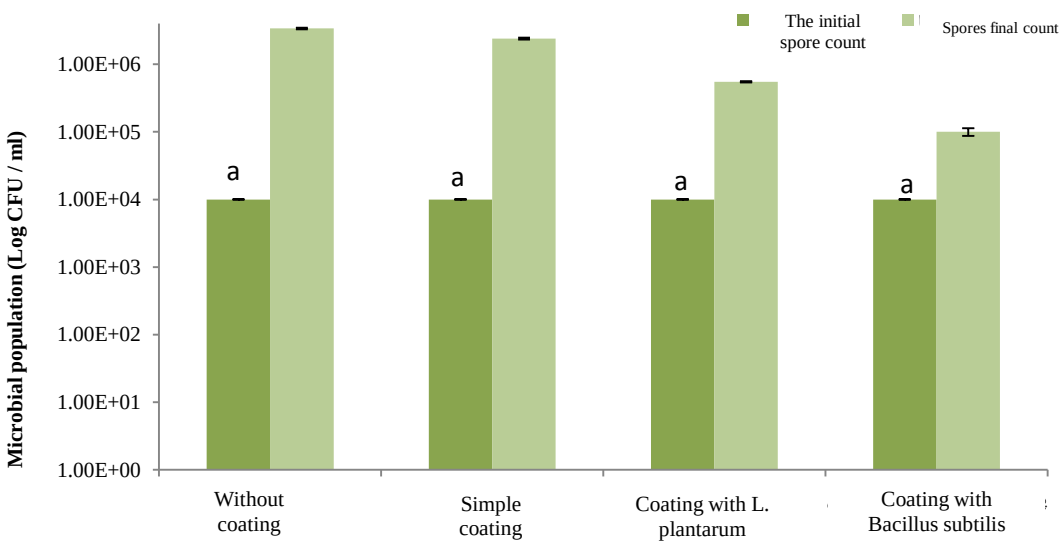

Fig 4. Spore counts on fresh pistachios during storage at 25°C for 30 day

3.5. Evaluation of the Viability of Bacterial Cells in the Aloe vera-based Coating

The changes in the microbial population of the bacteria applied in the film during storage for 4 weeks at 4°C are given in Table 2. Bacterial count after inoculation in the film-forming solution showed no adverse effect of the constituent compounds and film-producing conditions on the survival of microorganisms. After 7 days of storage at 4°C, two logarithms of the microbial population of both microorganisms were reduced. In the following weeks of storage, the microbial population of *Lactobacillus plantarum* decreased by one logarithm per week; this reduction continued until the end of storage time. On the other hand, *Bacillus subtilis* showed more resistance to the storage conditions and maintained its viability during 4 weeks of storage at 4°C to 6 Log CFU and after 7 days; this means that *it* can maintain its viability at a desirable level within 30 days compared to *Lactobacillus plantarum* in the film matrix. This demonstrates that temperatures, storage conditions, and microbial species are key in maintaining cell survival in the coating during the product storage time.

4. Conclusion

The results demonstrate that Aloe vera-based edible coating bearing *Lactobacillus plantarum* and *Bacillus subtilis* are appropriate choices to inhibit *Aspergillus flavus* growth in fresh pistachios. Among them, the latter, as a safe, economical, and effective microorganism, indicates a higher ability to prevent *Aspergillus flavus* growth, resulting in an elevation of the shelf life and quality of the final product, thus increasing its export revenue. Future studies should be carried out on the identification of antifungal compounds of *Bacillus subtilis* and the detoxification mechanism of microbial strains applied in the coating solution.

Aknowledgment: We specially thank Dr. Mohsen Farzaneh for their technical support throughout the project.

Funding: This study received any external funding.

Conflict of Interest: The authors declare that they do not have any conflict of interest.

Ethical Review: This study does not involve any human or animal testing.

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