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EFFICIENCY OF COMBINED EFFECTS OF LACTIC ACID BACTERIA (*Lactobacillus* SP.) AND HIGH OXYGEN ATMOSPHERE ON REDUCING THE POSTHARVEST DECAY OF STRAWBERRY FRUITS

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Abstract

Strawberry is one of the most delicate and perishable fruits which have a short postharvest life span estimated to be less than 5 days. It is prone to rapid dehydration, physiological disorders, bruising, mechanical injuries and infections caused by several pathogens which rapidly reduce the quality of the ripe fruit. The traditional application of chemical fungicides to control the postharvest diseases of strawberry fruits has several disadvantages, such as the adverse effects of pesticide residues on humans and favours the increase of pathogen-resistant strains. The present work used *Lactobacillus casei* as the bio-control agent along with the action of high oxygen atmosphere packaging to reduce the postharvest decay of strawberry fruits. Moreover, Lactic Acid Bacteria (LAB) is generally recognized as safe (GRAS) and usually matches all recommendations for food products. The dual culture assay results showed that the *Lactobacillus casei* did not inhibit the growth of fungal pathogen as no inhibition zone produced, but it slowed down their growth compared with the controls. Meanwhile, the *in-vivo* screening proven that no symptoms of infection seen on the fruits until the fifth day of storage for the strawberries which were treated with the biological control bacteria and highest concentration (80%) of modified oxygen. Hence, our results support the potential of *Lactobacillus casei* as a bio-control agent integrated with a high oxygen atmosphere against the postharvest rot of strawberry fruits.

INTRODUCTION

Strawberry (*Fragaria ananassa* Duchesne) is a non-climatic fruit that is succulent and delicate, in which it normally undergoes fungal spoilage after harvest. It is estimated that about 20-25% of the harvested fruits are decayed by pathogens during post-harvest handling even in developed countries [1]. Fruit rot disease caused by fungi is one of the major problems to strawberry cultivation and production reducing their quantity and quality and causing significant economic losses. Among the strawberry postharvest pathogens, *Botrytis cinerea* is known to be the most common and harmful [2]. *Alternaria* spp., *Aspergillus* spp., *Botrytis cinerea*, *Fusarium* spp., *Penicillium* spp., *Phytophthora cactorum*, *Rhizoctonia solani*, *Rhizopus stolonifer*, and *Sclerotinia sclerotiorum* are the most fungal isolates causing strawberry fruit rots in Egypt [3, 4]. In addition, there are ten common diseases of strawberries: anthracnose (black spot), grey mold, leaf spot, leaf scorch, *Gnomonia* fruit rot and leaf blotch, leaf blight, powdery mildew, *Fusarium* wilt, *Phytophthora* crown rot and *Aspergillus* fruit rot.

Previously, the synthetic fungicides method is known to be the most common and effective to control postharvest rot of fruits and vegetables. However, some of these are not authorized for postharvest treatment and several have been removed from the market due to possible toxicological risks (Directive 91/414/CEE of the EU). Also, the known traditional fungicides method is labelled as impractical nowadays as increasing of pathogen isolates that are resistant to one or more fungicides has been found [5]. The increasing demands by consumers for fruits and vegetables that are free of fungicides have contributed to the development of alternative methods by researchers and the food industries for controlling postharvest decay of fresh products.

An alternative to chemical application is the use of microorganisms as bio-control agents. In recent years, lactic acid bacteria (LAB) have attracted much attention as antimicrobial agents. Several important antimicrobial compounds and active substances produced by LAB together with hydrogen peroxide and carbon dioxide, such as lactic acid, propionic acid, antibiotic and bacteriocin [6]. The bacteriocin molecule helps to inhibit the deterioration in food and thus acts as a potential biological protective agent to prolong the shelf life of food [7]. Meanwhile, the application of LAB as a bio-control agent is very recommended due to the ability to prevent the development of molds [8]. The bacteria also have a wide growth range, which allows their application under different conditions, including refrigeration temperatures. The known species of lactobacilli which have been identified as potential antifungal cultures and able to extend the shelf life of various products, including *L. casei, L. rhamnosus, L. paracasei, L. sanfranciscensis, L. fermentum, L. helveticus, L. sakei, L. rossiae, L. amylovorus, L. harbinensis, L. brevis,* and *L. spicheri* [9, 10].

Enhancement of biological control could be obtained by combining organic and inorganic additives. Also, the biological control is only effective when the concentration of the antagonist is at the optimum level, but the efficacy is not as useful as fungicides [11]. Several studies had been done about the modest efficiency of the biological control when applied alone, and the efficacy of combining antagonists with other postharvest treatments [12]. The combination of different preservation methods such as modified atmosphere packaging, low-temperature storage, or the additional preservation methods may be an excellent way to preserve the original quality attributes of fruits and vegetables. Elevated oxygen atmosphere is suggested as a better alternative to the traditional low oxygen and high carbon dioxide modified atmosphere packaging to maintain quality and safety [13]. Also, a high oxygen atmosphere able to inhibit the growth of microorganisms and prevent undesirable anoxic fermentation [14].

Even the presence of the evidence about *L. casei* antimicrobial effect on plant pathogens, there is not much research that has been performed on their potential on strawberry postharvest disease management. Because of that, this study was performed to determine the effects of *L. casei* against the fungal pathogen in vitro and in vivo along with the action of high oxygen atmosphere packaging to reduce the postharvest decay of strawberry fruits. Besides, the performance of these combined methods in preserving the quality and freshness of strawberry fruits or prolong the shelf-life were also discussed.

MATERIALS AND METHODS

Sample Collection

Healthy and diseased samples of strawberry fruits were purchased from a market. Healthy strawberries of uniform size and color and free from wound and rot were chosen. Meanwhile, naturally rotted of strawberry fruit samples showing disease symptoms, such as discoloration, a circular black spot, slightly sunken, and brown water-soaked lesion were selected. Before each test, the fruits were washed with sodium hypochlorite solution (1% active chlorine), then rinsed with sterile distilled water and left to dry by using sterile filter paper.

Isolation of Lactic Acid Bacteria

The bio-control agent bacteria, *L. casei* was isolated from commercial Yakult. Fold serial dilutions (10^{-1} – 10^{-5}) of bacteria suspension were done. A total of 0.5 ml of aliquots from five-fold dilutions of the suspension were used for isolating the antagonist bacteria by the spread plate method and incubated for 3 days at 37ºC [15]. The selective deMan, Rogosa and Sharpe (MRS) agar media were used to grow the bacteria. After incubation, single colonies of the bacteria were counted by using the colony counter before transferring onto new fresh MRS agar plates to obtain single and isolated bacterial colonies. Then, the cultures were incubated again for 3 days at 37ºC. Lastly, the bacteria cultures were grown on MRS agar and maintained at 4ºC until used.

Isolation of Fungal Pathogen

The Potato Dextrose agar (PDA) medium was used to culture the fungal pathogen, in which 5 mm^2 pieces of infected strawberry parts were cut and placed on the fresh PDA agar plate [16]. All the plates were incubated for 5 days at 37ºC. The sub-culturing process was repeated twice to ensure only pure isolates of fungal pathogens were grown.

Preparation of Aqueous Bacteria Suspension and Conidial Suspension of Pathogen

The selected bacteria isolate was grown on MRS agar at 37ºC for 3 days. A loopful of each culture was then transferred to a universal bottle containing 7 ml of MRS broth and incubated for 48 hours at 37ºC. The isolate was sub-cultured for at least two times. At the time of use, the

bacterial suspension was adjusted to approximately 10^8 cfu ml^{-1} [17].

The isolate of the fungal pathogen was grown on PDA agar at 37°C for 5 days. Spores were subsequently harvested by flooding the surface of the media with distilled water and gently stirred using a bent glass rod to dislodge the spores [18].

DNA Extraction, Quantification and Purification

The antagonist bacterial and fungal pathogens were identified by using 16S ribosomal RNA (rRNA) gene sequence analysis [15]. DNeasy Blood & Tissue kit (Qiagen) was used to extract the DNA of bacteria. DNA extraction of the fungal pathogen was extracted by using DNeasy Plant & Mini kit (Qiagen). The extracted DNA was quantified and purified using a NanoDrop ND-1000 (NanoDrop Technologies). Then, the extraction products were separated by electrophoresis using 0.8% (w/v) agarose gel electrophoresis and viewed under UV transilluminator AlphaImager® (AlphaInnotech, California).

PCR Amplification

Polymerase chain reaction (PCR) was carried out according to the protocol provided in PCR Amplification kit (Qiagen). The 16S rRNA gene fragments were amplified using 1 µl of each cell suspensions as template and universal primers. The F-27 (5'-AGAGTTTGATCCTGGC TCAG-3') and R-1492 (5'-TACGGYTACCTTGTTACGACTT-3') were the universal primers for bacteria. For the amplification of pathogen, the universal primers of F-ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and R-ITS4 (5'-CCTCCGCTTATTGATATGC-3') were used. The master mix contained approximately 500 ng of total bacteria or fungal pathogen DNA, 2 µl of 10x PCR buffer, 2 µl of 25 mM $MgCl_2$, 0.4 µl of 10 mM dNTPs mix, 0.2 µl of each primer (10 mM), 0.4 µl of DNA *Taq polymerase* (5 U/µl) and enough Milli Q water until the final volume of the mixture was 20 µl [19].

Each sample was subjected to an initial denaturation for 1 minute at 94°C followed by 30 amplification cycles of denaturation at 94°C for 1 minute, annealing gradient from 42°C-50°C for 45 seconds and extension at 72°C for 3 minutes [15, 19]. After the final extension at 72°C for 7 minutes, the samples were kept at 4°C until further used. Six µl of each PCR product was electrophoresed on 2% (w/v) agarose gel containing 1.3 µl GoldView™ Nucleic Acid Stain (Lonza, USA) at 90 V for about 90 minutes. The gel was photographed on a UV transilluminator. The total PCR products were purified by using PCR Purification kit (Qiagen) by following the supplier's instructions.

DNA Sequencing and BLAST

The purified DNA fragments of antagonist bacterial and fungal pathogens were sent to the third party company for further sequencing. When the sequencing of purified DNA was obtained, the sequence was analyzed using BLAST program selection, a free genomic database program. The regions of similarity between sequences were detected by the BLAST program and compared the nucleotide to the sequence database and the statistical significance of the matches was calculated (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Dual Culture Assay

One 6 mm diameter of fungal pathogen agar plug was placed at the center of MRS medium in a petri dish [20]. Bacterial isolate was streaked on MRS medium at a distance of 2.5 cm from the fungal pathogen agar plug. Plates were incubated for 5 days at 37°C. The inhibition of mycelial pathogen growth by the antagonist bacteria was observed for 5 days. The control plate without the bacterial isolate was also prepared for comparison of pathogen growth. Then, the percentage of mycelial growth inhibition was calculated using the formula: Growth inhibition (GI) = $(R_1-R_2) / R_1 \times 100$; R_1 = Distance (measured in mm) between the point of implant of the fungal disc and the side of the Petri plate; R_2 = Distance of fungal growth from the point of inoculation to the colony margin in the direction of the antagonist bacteria.

***In Vivo* screening**

Four healthy strawberries with no visible wound and rot were washed in sodium hypochlorite solution (1% active chlorine). The fruits were dried using sterile filter paper after rinsed with distilled water. Then, each fruit was weighed (weight before treatment) and soaked in a bacterial suspension (10^8 cfu ml^{-1}) for about 12 seconds [20]. The fruits were left to dry for 1 hour on the sterile filter paper. Later, as the pathogenic inoculum, solutions prepared by disruption of fungal mycelia developed in the liquid PDA medium and adjusted to a density of 10^6 spore ml^{-1} and injected into each fruit [21]. The fruit was placed inside a beaker (covered with aluminium foil). Four anaerobic jars with different candle lengths (A: 20% O_2; B: 40% O_2; C: 60% O_2 and D: 80% O_2) were prepared. Each anaerobic jar was placed with a beaker contained fruit, then the candles were lighted up and closed the lid of jars. All jars were kept in the refrigerator after the light of the candles extinguished. Measurement of fruit weight were taken every day until five days of treatment. Also, the color and changes in the physical appearance of fruits were observed and recorded [22]. Controls were designed as fruit + *L. casei* and fruit only (without treatment). This experiment was done in triplicate.

RESULTS AND DISCUSSION

Isolation of the bio-control agent bacteria was carried out from a commercial Yakult. Yakult contains *Lactobacillus casei* strain Shirota. After done the fold serial dilutions (10^{-1} – 10^{-5}) of suspension, the antagonist bacteria were isolated on the MRS agar by spread plate method. Bacteria concentration at 10^{-4} dilution showed growth of white, round and single colonies (Figure 1). Single and pure colony of the *Lactobacillus* species was sub-cultured onto new fresh MRS agar for further identification. Meanwhile, the isolation of fungal pathogen was performed from infected strawberry fruits purchased from a market. At an early stage (day 3 of incubation), the colonies showed a green color followed by a dark green color on day 4 of incubation. At day 5 of incubation, the colonies turned black which indicated that it reached the maturing stage and macroscopically identified as an *Aspergillus* spp. (Figure 2) [23].

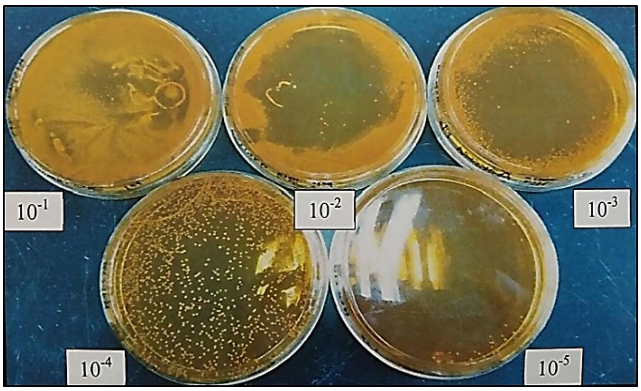


Figure 1. The growth of *Lactobacillus* species on MRS agar at day 3 of 37°C incubation

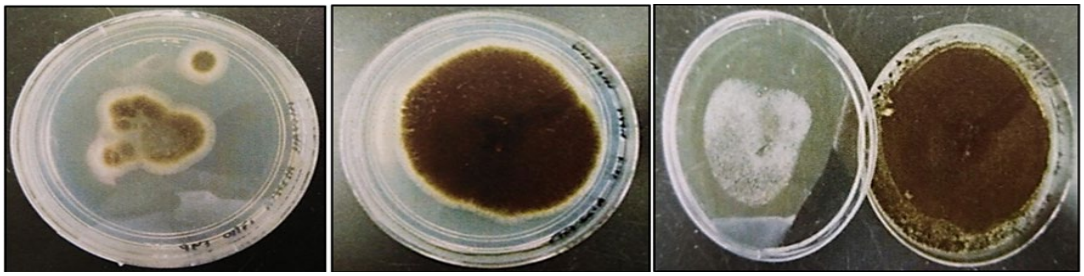


Figure 2. The growth of fungal pathogen on the third, fourth and fifth days (left to right) of 37°C incubation.

A single band appeared in the gel on lanes 2 to 7 with a similar band size. When compared to the 100 bp DNA ladder, the size of the amplified product of antagonist bacteria DNA was 1,500 bp which also the same as the positive control (*E. coli*) (Figure 3). Hence, this implied that the amplified product of antagonist bacteria DNA was of the expected size of DNA fragment. The expected range of amplified product of *Aspergillus* spp. DNA was between 565 bp to 613 bp [23]. Based on the gel observation (Figure 4), a single band with a size of 600 bp appeared on lane 7. The PCR products of both samples were purified and sent for sequencing. But, only the sequencing result of antagonist bacteria was confirmed due to the low concentration of the purified fungal pathogen DNA. Based on the BLAST result, it can be concluded that the isolated *Lactobacillus* species is a *Lactobacillus casei* bacteria (Figure 5). The maximum identity was 96% which prove that the isolated *Lactobacillus* species has 96% identity to the species data in the Genbank. The highest maximum score of 1507 and zero E-value further indicated that the result was accurate.

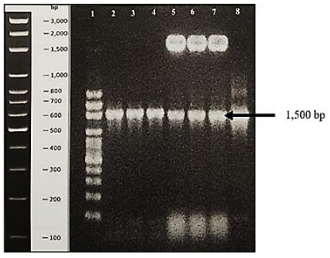


Figure 3. PCR amplifications of antagonist bacteria under UV transilluminator. Lane 1 is the 100 bp DNA ladder; lanes 2-7 are PCR products; lane 8 is the positive control *(E. coli*).

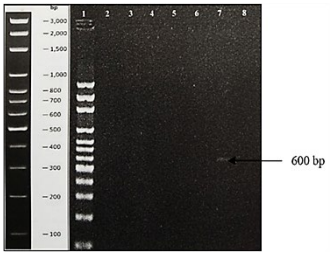


Figure 4. PCR amplifications of fungal pathogen under UV transilluminator. Lane 1 is the 100 bp DNA ladder; lanes 2-7 are PCR products; lane 8 is the negative control (sterilized distilled water).

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Lactobacillus casei strain NM60-1 16S ribosomal RNA gene, partial sequence	1507	1507	98%	0.0	96%	HM218266.1
Lactobacillus paracasei strain FT232 16S ribosomal RNA gene, partial sequence	1502	1502	98%	0.0	96%	KM207837.1
Lactobacillus casei strain MTZH493 16S ribosomal RNA gene, partial sequence	1502	1502	98%	0.0	96%	KJ804189.1

Figure 5. BLAST result for *Lactobacillus* species.

Screening and evaluation of antagonist bacterial activities were done by calculating the percentage of pathogen inhibition growth based on the formula given in the methodology. Based on the observation, it showed that the *L. casei* does not inhibit the growth of *Aspergillus* spp. as no inhibition zone produced, but it slowed down the fungal growth compared with the control (Figure 6). The structure of fungal pathogen consists of conidiophore, which held the vesicle, phialides and conidiospores. The observation under the microscope showed that on the third day of incubation, the spores were detached from the conidiophore (Figure 7). On the fifth day of incubation, the conidiophore was absent from the observation and only the sporangium was seen. It can be concluded that the processes of *Aspergillus* spp. to become fully developed and matured were disrupted by the *L. casei*, which slow down their growth development and eventually affect their fungal activity. The *in vivo* screening revealed that for the control strawberries (without treatment), dark spots started to appear in the middle of the fruit on the 2nd day and continued to develop deep sunken spots until the 5th day of storage. For the fruits treated with bio-control agents only, no symptoms of fruit decay can be seen until the final day of treatment except that the color of fruits slightly turned darker (Figure 8). The treated strawberries with *L. casei* and 20% O_2, dark spots and sunken spots appeared on the 3rd and 5th day, respectively. When 40% O_2 was applied to the fruits, the delay of infection was found in which dark spots appeared on the 4th day and continued until the 5th day without the appearance of usual sunken spots. Meanwhile, no symptoms of infection observed for the fruits that were treated with 60% O_2, but the fruits started to turn darker on the 3rd day. Lastly, the highest strawberries atmosphere of 80% O_2 maintained a healthy and red color of the fruits until the 5th day of storage with no symptoms of infection seen. Hence, this study proved that the efficiency of combined methods is the highest especially by using 80% O_2, in which the quality of the fruits in terms of color maintained healthy and the possibility to prolong the shelf-life of strawberry fruits could be achieved. Strawberries stored at high oxygen atmospheres (>40%) showed higher antioxidant capacity, total phenolics, less decay and longer postharvest life than those stored in air [24]. Strawberries are good sources of natural antioxidants. An antioxidant is a molecule that inhibits the oxidation of other molecules. Oxidation is a chemical reaction that

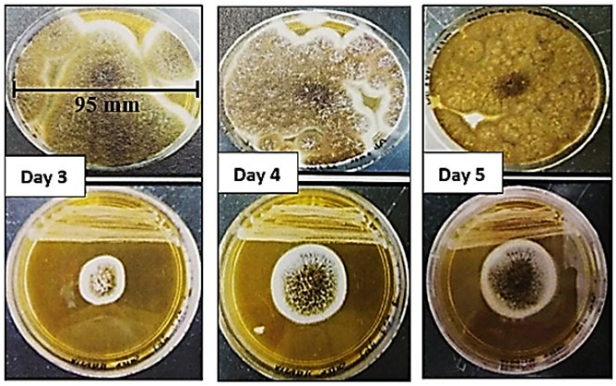
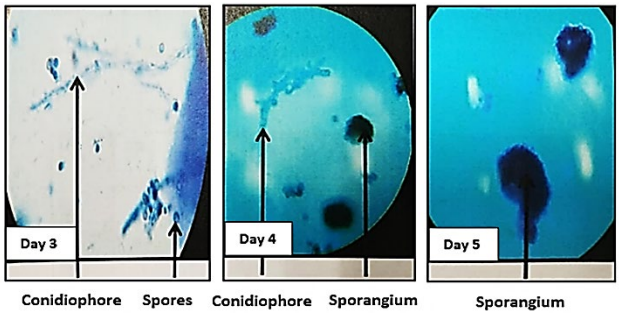


Figure 6. The dual culture assay (bottom plate) of *Lactobacillus casei* against the *Aspergillus* spp. compared with the control (upper plate).



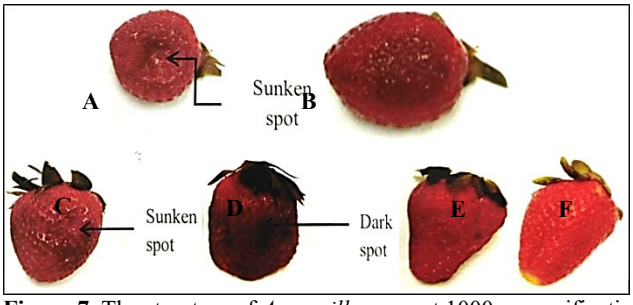


Figure 7. The structure of *Aspergillus* spp. at 1000x magnification during the dual culture assay.

Figure 8. *In-vivo* screening of *L. casei* with oxygen atmosphere treatment against *Aspergillus* spp (C: 20% O_2; D: 40% O_2; E: 60% O_2 and F: 80% O_2). Controls; A: fruit only (without treatment) and

B: fruit + *L. casei*. *Note: The strawberry fruits condition on the fifth day of treatment.*

transfers electrons or hydrogen from a substance to an oxidizing agent. High oxygen concentration will cause an oxidation reaction to take place and produces free radicals. Free radicals can start a chain reaction that can cause damage or death to the cell. Antioxidants terminate these chain reactions by removing free radical intermediates and inhibit other oxidation reactions. This situation prevents the decay of the fruit and reduces water losses [25]. It is also suggested the use of 80% O_2 concentration to reduce significantly mycelium growth and the amount of mold spores [26].

Figure 9 shows that the control strawberries (without treatment) have the highest water loss which was 1.45%. When using only one treatment (*L. casei*), the efficiency to reduce water loss was lesser than the combined treatments. Meanwhile, the increase in oxygen concentration reduced the water loss or loss of fruit weight. The 20% O_2 strawberries atmosphere recorded the greatest loss in weight of fruit at 0.38%, followed by 40% O_2 strawberries atmosphere at 0.31%, 60% O_2 strawberries atmosphere at 0.18% and 80% O_2 strawberries atmosphere showed the least loss of weight at 0.13%. Probably bacterial fruit treatment further helped to reduce the weight loss by coating the fruit surface thereby reducing the water loss. A study done previously stated that the construction of a hydrophobic film that was formed from a continuous matrix around the product was reported to be bacterial exopolysaccharides [27]. Besides that, the use of *L. casei* which is a Gram-positive bacterium is better protected from the toxic effect of oxygen metabolites because of their thick peptidoglycan layer in their cell wall outside the cell membrane.

In conclusion, since the *L. casei* only slows down the growth of Aspergillus spp. (no inhibitory activity towards pathogen), thus considerable efforts are needed to identify new Lactobacillus species with the capacity to act as bio-control agents. The application of oxygen meter rather than candles to control or modify the oxygen concentration is more practical and efficient.

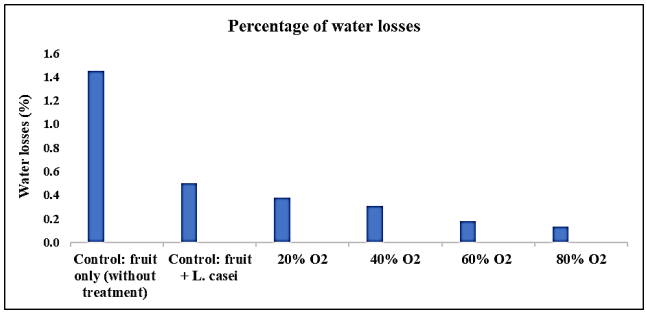


Figure 9. The percentage of water losses versus oxygen concentration. *Note*: Water losses (%) = [(weight before treatment – weight after treatment)/ weight before treatment] x 100

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this manuscript.

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