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Inclusion of Antifungal and Probiotic *Lactiplantibacillus plantarum* Strains in Edible Alginate Coating as a Promising Strategy to Produce Probiotic Table Grapes and Exploit Biocontrol Activity

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Abstract: The use of Lactic Acid Bacteria (LAB) for probiotic enrichment of minimally processed fruit is a well-established practice in literature. In addition, several LAB demonstrated a strain-specific ability to control harmful microorganisms and decay agents, improving shelf-life, maintaining quality and promoting the safety of fruits and vegetables. Edible coatings can help modulate phenomena of gas exchange and water loss by fruits, representing protection from physical damage and spoilage phenomena linked to oxidation and the development of undesired microorganisms. At the same time, the coating can represent an innovative delivery matrix for the LAB strains of potential interest to improve safety and quality in the postharvest management of fruits. In this work, five *Lactiplantibacillus plantarum* strains, previously characterized for their probiotic and antifungal activity, have been incorporated into a sodium alginate coating to develop edible probiotic coatings with antifungal properties for table grapes cv. Italia. The transfer of probiotics and their survival were evaluated by comparing coated and uncoated table grapes during 14 days of cold storage at 4 °C. The alginate edible coating increased the number of probiotics transferred to the surface of the berries from about 5 to more than 7 Log CFU/g, with a crucial impact on the potential functional attributes of the final product. The ability of the functionalised coatings to counteract the decay development was evaluated on table grapes berries artificially contaminated with *Aspergillus niger* CECT 2805. A significant reduction of lesion diameter was observed in alginate coating with *L. plantarum* 11-A. The lesion diameter reduction was also accompanied by the reduction of the symptoms of infection, such as browning around the wound. These results suggest the application of selected strains of *L. plantarum* as promising bio-resources to enhance the overall value of ready-to-eat fruits and vegetables, particularly in combination with edible coating as a carrier matrix. While a strain-dependent effect was not detected with respect to the improvement of the number of cells in the edible coating, a variability depending on the biotype used was detected for the properties linked to biocontrol, suggesting that the inclusion in edible packaging may represent an innovative criterion in the selection of lactobacilli to be applied postharvest.

Keywords: lactic acid bacteria; lactobacilli; *Lactiplantibacillus plantarum*; table grapes; fruit; antimicrobial; probiotic; edible coating; postharvest; *Aspergillus niger*; spoilage

1. Introduction

Probiotics are live bacteria that, when administered in sufficient quantities, provide health benefits to the host [1,2]. Consumers typically ingest probiotics from dairy, pharmaceutical, and fermented foods. On the other hand, dairy products do not correspond to the dietary needs of specific customer categories, such as vegetarians and lactose-intolerant consumers [3]. As a result, the development of non-dairy alternatives based on fruit and vegetables, such as probiotic fresh-cut fruits, could serve as a preference vector for these consumers [4]. 'Minimally processed', 'ready-to-eat', 'fresh-cut', and some other designations indicate fruits ready for consumption [5], categories that have growing popularity attributable to their convenience, sensory quality, excellent nutritional value, and ability to retain freshness [6]. Recently, probiotic addition was proposed for a growing number of fresh-cut fruits, such as apples, pears, cantaloupes, pineapples, carrots and blueberries [7–12]. However, several authors reported that specific features of the fruit matrices could affect the probiotic viability during shelf life and that the metabolic activity of the strain utilized could have a detrimental effect on the sensory characteristics of the fruit [8]. In this light, the use of edible coating based on biopolymers could act as an immobilizing agent to limit the metabolic activity and the proliferation of the probiotic cells added [13,14], but also for extending the shelf life of fresh-cut fruits [15], as it could act as a barrier to water and gas, and reduce the water loss and oxidative reactions of the fruits [16–18]. Among the most appreciated features of the probiotic strains selected for the application in the fresh-cut sector, there is the ability to control harmful microorganisms and decay agents. In this context, the use of antagonistic microorganisms, such as Lactic Acid Bacteria (LAB), has assumed international relevance as a promising eco-friendly alternative to chemical interventions [19], with the aim of sustainable improvement of the shelf-life, quality, and safety of fruits and vegetables [20–22].

Lactiplantibacillus plantarum is a lactic acid bacterium belonging to the heterogenous class of lactobacilli. This bacterium is an extremely widespread species in the agro-food sector due to its outstanding biological versatility. Among the most interesting characteristics of this microorganism it is possible to include the probiotic features and antimicrobial properties, both strain-related characteristics [23–25]. These are two interconnected attributes, as among the selection criteria of a probiotic microorganism, there are antimicrobial properties with respect to pathogenic bacteria, with the aim of supporting intestinal health. Different strains of *L. plantarum* have found application in the postharvest of fruits and vegetables [21,26–28], with particular reference to the issues of biocontrol and improvement of functional quality, supporting the interest in this microorganism as a model lactic acid bacteria for this kind of applications. Table grapes are non-climacteric fruits, particularly perishable after harvest, which tend to deteriorate due to water loss, oxidation, and fungal colonization. Several treatments were proposed to maintain the postharvest quality of table grapes [29] and to limit the decay induced by its most important fungal spoilage, *Botrytis cinerea* [29,30]. However, colonisation by other fungal species was reported for table grapes [31]. Among those, *Aspergillus* genus is one of the most dangerous filamentous fungi because they can colonize a wide range of food commodities and can spread and propagate in the storage phase. This genus is also responsible for safety concerns regarding allergenic reactions and the production of mycotoxins, such as aflatoxins and ochratoxin A [32].

In this work, five *Lactiplantibacillus plantarum* strains, previously characterised for their probiotic and antifungal activity, have been incorporated into a sodium alginate coating to develop edible probiotic coatings for table grapes cv. Italia. In addition, the ability of functionalized coatings to counteract the decay development was evaluated on table grapes berries artificially contaminated with *Aspergillus niger*.

2. Materials and Methods

2.1. Microbial Strains and Growth Conditions

Five *L. plantarum* strains isolated from fruits and vegetables previously characterized for their probiotic and antimicrobial activity were selected for this study [33,34]. Lactic Acid Bacteria were routinely cultured in MRS broth (Oxoid, Basingstoke, United Kingdom) at 30 °C.

The filamentous fungus *Aspergillus niger* CECT 2805 from cryopreserved cultures was propagated on Potato Dextrose Agar at 24 °C for five days. Fungal spores suspension was prepared by brushing the plate surface with saline solution (0.86% NaCl) supplemented with 0.01% Tween 80 using a sterile swab, storing the suspension at 4 °C for short-term uses. Fungal spores concentration was determined by plating serial dilution on PDA plates and adjusted to approximately 1 x 10^6 spores/mL.

2.2. Preparation of the Probiotic Coating-Forming Solutions

The probiotic coating-forming solutions were obtained as reported by Alvarez et al. [7]. The coating-forming solution consisted of 2% (*w/v*) alginate (Sigma-Aldrich, St. Louis, MO, USA) with 1.5% (*w/v*) glycerol as a plasticizer. After complete dissolving, the coating-forming solution was autoclaved at 121 °C for 15 min and cooled down until room temperature. *L. plantarum* strains were grown overnight in MRS broth, washed twice and resuspended in coating forming solution to obtain a final concentration of ~ 10^9 CFU/mL. Inoculum concentration was checked by plating appropriate dilutions onto MRS agar.

2.3. Preparation of Probiotic-Coated Table Grape Berries

Healthy table grapes (cv. Italia) were purchased from a local retailer, sanitized by dipping for 1 minute in 0.01% (*w/v*) sodium hypochlorite (NaOCl), rinsed twice with sterile demineralized water, and dried under a laminar flow hood. Berries were dipped into the probiotic coating solution and then in the hardening solution (2% *w/v* $CaCl_2$) both for 30 sec. Uncoated grapes were dipped in saline solution containing the same amount of probiotics and then in hardening solution. After drying, probiotic table grapes berries were packed in plastic containers (five berries each) under passive atmosphere and stored at 4 °C for 14 days to simulate commercial shelf-life. Each treatment was performed in triplicate.

2.4. Probiotic Viability

The survival of the probiotic strains in coated and uncoated berries during the simulated shelf-life was evaluated. Analysis was performed after 0, 7 and 14 days of storage at 4 °C. Samples were mixed (1/10 *w/v*) with sterile saline solution and homogenized for three minutes in a stomacher blender. Then, serial 10-fold dilutions were plated on MRS agar. Viable counts expressed as log of colony forming units per gram (LogCFU/g) were determined after incubation of the plates at 30 °C for 48 hours.

2.5. Fruit Decay Assay

Probiotic-coated table grape berries were prepared as described above. Then, artificial wounds were performed using a sterile needle to make 3 mm deep and 3 mm wide wounds (four wounds for each acinus) along the equatorial areas of the berries. Each wound was inoculated with 10 μL of *A. niger* spores suspension (~ 10^6 spores/mL). After drying in a laminar flow hood for about 1 hour, table grapes berries were packed as above. Five berries were wound-inoculated for each probiotic treatment. The plastic containers were maintained at 24 °C for 3 days, in order to create favorable conditions for the onset of post-harvest pathology. The development of the fungus was monitored daily by visual analysis.

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2.6. Sensorial Quality Analysis

A group of ten trained panellists performed the sensory evaluations of artificially contaminated table grape berries after 3 days of shelf life at 24 °C. Panellists received training in order to identify and rate the non-tasting quality attributes prior to evaluations. Positive descriptors, such as appearance, colour, freshness, firmness, and overall acceptance, were evaluated using a hedonic scale from 1 to 5, where 1 = not present/very low/not typical, to 5 = very pronounced/very typical of fresh fruits. Negative descriptors, such as off odour and mould occurrence, were ranked from 1 = 0% mould presence/odour to 5 = 100% mould presence/odour. In both cases, the value 3 was fixed as the limit of marketability.

2.7. Statistical Analysis

One-way analysis of variance (ANOVA) was performed by using SAS statistical computer package (SAS Institute, Cary, NC, USA). Significant differences in bacterial viability were determined using Fisher's Least Significant Difference (LSD) test with $p < 0.05$ as the minimal level of significance. Significant differences in lesion diameter were determined by post hoc Tukey's Honestly Significant Difference (HSD) test with $p < 0.05$ as the minimal level of significance.

3. Results and Discussion

LAB belonging to the genus *Lactiplantibacillus* are typically found in association with fruits and vegetables [35], and they are considered natural competitors of the undesired microflora responsible for spoilage and decay of fruits and vegetables, such as phytopathogenic bacteria and filamentous fungi [36]. In particular, the species *Lactiplantibacillus plantarum* also assumed a relevant role as biocontrol agent because it is already adapted to fruit environments and their related stressors, thus simplifying its application for industrial purposes [36]. The competition between LAB and filamentous fungi is mediated by a plethora of mechanisms, including the competition for nutrients, the secretion of metabolic byproducts (i.e. organic acids) and the production of active compounds such as peptides and VOCs, but also for synergistic mechanisms among those [37,38]. In this paper, five strains of *Lactiplantibacillus plantarum* isolated from wild plant matrices of the Mediterranean area (i.e. aloe, carob, blackthorn), considered as unconventional ecological niches due to their restricted use in food industries and already characterized for probiotic [33] and antimicrobial activity ([34]; De Simone et al. *unpublished results*), were selected for their ability to inhibit the growth of *Aspergillus niger* CECT 2805. Different modes of action and synergies among the antimicrobial features were previously identified as responsible for the detected activity. In fact, the five strains are able to produce different organic acids, including lactic, acetic and 3-phenyllactic acid, but also volatile organic compounds, such as high amounts of 2-undecanone and 2-nonanone, which are well known for their antifungal activity [34]. For these reasons, the strains were chosen for probiotic fortification and for application as biopreservatives on ready-to-eat perishable fruits using table grapes cv. Italia berries as a model fruit. In this context, the use of coating was chosen as an immobilizing agent with the aim of increasing the number of probiotic cells delivered by the berries and uniformly distributing the biocontrol strains on the surface of the fruits, but also as a combined treatment to ameliorate the effect of bioprotection. In this work, an edible coating based on sodium alginate and glycerol was chosen because these chemicals are already used as food additives, with the codes E401 and E422, respectively.

3.1. Probiotic Viability on Table Grapes Berries during Shelf Life

The viability of probiotic *L. plantarum* strains on table grapes berries included or not into edible coating during 14 days of shelf life at 4 °C is shown in Figure 1. At day 0, the alginate edible coating increased the number of probiotic cells transferred to the surface of the berries, which was generally increased from about 5 to more than 7 LogCFU/g for all the strains used in this work. A greater number of probiotic living cells for coated berries with respect to uncoated berries was recorded on all sampling days. However, in uncoated berries, the number of living cells remained stable during the 14 days, whereas a reduction of about 1 Log CFU/g was detected in the coated berries. No difference was found between the *L. plantarum* strains at sampling days 0 and 7 in both conditions. At 14 days, *L. plantarum* 11-A and CZ-97 showed higher viability in uncoated grapes, with about 5,7Log CFU/g, whereas, in coated berries, *L. plantarum* 11-A was found significantly different, with 6,7 Log CFU/g.

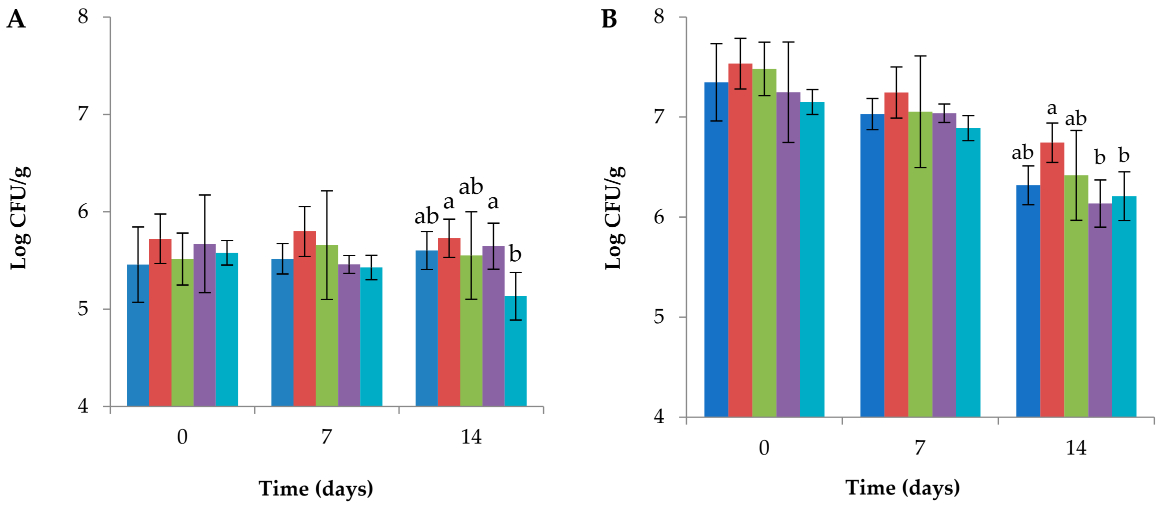

Figure 1. Viability of probiotic *L. plantarum* strains in ready-to-eat table grapes berries during 14 days of storage at 4 °C, applied through saline solution (**A**) or into edible coating (**B**). Values are means and standard deviation of three biological replicates. Statistical analyses were carried out by one-way ANOVA test ($p < 0.05$) followed by Fisher's Least Significant Difference (LSD) test. *L. plantarum* 10A (blue); *L. plantarum* 11A (red); *L. plantarum* CB56 (green); *L. plantarum* CZ97 (purple); *L. plantarum* CZ103 (light blue).

Without edible coating, Lappa et al. [39] could transfer a slightly lower quantity of *L. plantarum* than what we observed in uncoated grapes. Whereas, in our precedent work, *L. plantarum* MEP3 had better adhesion ability on uncoated table grapes berries, with approximately 7 Log CFU/g of viable cells transferred to the surface of the berries, that remained stable during shelf life [40], indicating the need to develop new strategies to increase the adhesion of probiotic bacteria with limited ability to colonize the surface of the fruits. In addition, the LAB viability observed in ready-to-eat table grapes was slightly lower than in other fresh-cut fruits [8,9,40,41]. For this reason, it should be considered that the decreased ability to colonize the fruit's surface may be due to the structural and chemical characteristics of the cuticle of grape berries, which may prevent microorganisms from adhering to and remaining on the fruit [40]. However, probiotic intake advantages can be gained from foods that contain at least 6-7 Log CFU of viable bacteria per gram of product [42]. Thus, the use of edible coatings could be considered an advantageous option to obtain probiotic ready-to-eat fruits. In fact, the content of viable probiotic cells in uncoated table grapes berries does not reach the requirement limit to be considered as beneficial. In this light, the use of edible coating gave the possibility to enhance of about 2 Log the quantity of probiotic bacteria transferred to the berries' surface, thus fulfilling the requirement limit of 6-7 LogCFU/g also at the end of shelf life. In addition, assuming that the mean weight of one grape berry is between 8 and 12 grams on average, and considering that an estimated portion of fresh fruit in a single meal could be represented by 5 - 10 berries, with a quantity between 80 – 100 g of product, the concentrations of probiotic for a single portion of ready-to-eat table grapes berries should be estimated to more than 8 LogCFU also at the end of shelf life, tailoring this matrix as a functional food.

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3.2. Fruit Decay Assay

Based on previous characterization of antifungal activity against *Aspergillus niger* CECT 2805 [34], it was also hypothesized that probiotic *L. plantarum* strains could act as a preventive treatment against fungal contamination in table grapes berries. For this reason, the bioprotective potential of the probiotic strains applied to edible coatings was further investigated against the same target when artificially wound-inoculated. The lesion diameter and the symptoms of infection of the table grape berries subjected to different probiotic edible coating treatments are shown in Figure 2.

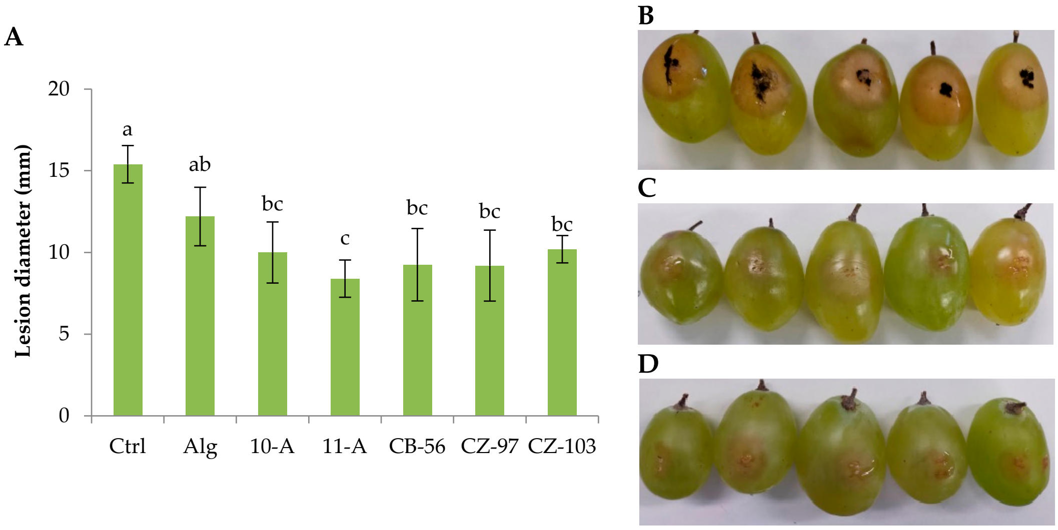

Figure 2. Lesion diameters of table grapes berries artificially contaminated with *A. niger* CECT 2805 after 3 days of shelf-life at 24 °C (**A**). Ctrl: Untreated control. Alg: Alginate coating. 10A: *L. plantarum* 10A /Alginate coating. 11A: *L. plantarum* 11A/Alginate coating. CB56: L. *plantarum* CB56/Alginate coating. CZ97: *L. plantarum* CZ97/Alginate coating. CZ103: *L. plantarum* CZ103/Alginate coating. Data are the means ± SD of five replicates. Statistical analyses were carried out by one-way ANOVA test ($p < 0.05$) followed by Tukey's multiple comparison test. Table grapes cv. Italia berries artificially contaminated with *A. niger* CECT 2805: Untreated control (**B**), Alginate coating (**C**), *L. plantarum* 11A/Alginate coating (**D**).

As reported in previous studies, the alginate coating treatments effectively reduced postharvest deterioration and reduced quality parameters, such as weight and firmness losses, total soluble solids, titratable acidity, and colour [43,44]. Our work only investigated the potential to reduce the lesion diameter caused by fungal inoculation in artificially performed wounds. In this case, when alginate coating alone (without probiotic cells) was compared to uncoated control, a significant effect on lesion diameter reduction was observed, with a lesion diameter from 15,40 ± 1,14 to 12,20 ± 1,79 mm for uncoated and alginate coated berries, respectively (Figure 2A). The lesion diameter reduction was also accompanied by the reduction of the symptoms of infection, such as browning around the wound and sporulation (Figure 2B,C). This is consistent with previous literature in this topic and confirms the positive effect of edible coatings to limit the physiological damage of the berries. However, among the probiotic coatings, most of the strains showed the same behaviour, and only slight differences were found between most of them and the alginate coating alone. In fact, four out of five strains showed the same statistical significance level, with values of lesion diameter ranging from 10,00 ± 1,87 to 9,20 ± 2,05 mm, for *L. plantarum* strain 10-A and CZ-103, respectively. Differently, the coating treatment with *L. plantarum* 11-A showed a higher reduction in lesion diameter, with values of 8,40 ± 1,14 mm, which was significantly different from the other strains and from the alginate coating alone. In addition, the symptoms of infection were also lower, with the absence of sporulation and only a limited browning around the wound, which partially healed (Figure 2D). Different authors have previously reported the protective effect of living cells of L. plantarum against fungal decay caused by *Aspergillus* species [39,40]. At the same time, the inhibitory effect of the CFS of *L. plantarum* strains was assessed in table grapes against *Pseudomonas syringae* pv. *syringae*, and *Botrytis cinerea* [45]. This suggests the high potential of this LAB species as a broad-spectrum biocontrol agent for table grapes. However, to the best of our knowledge, the biocontrol effect of edible coatings functionalized with antifungal strains of *L. plantarum* against fungal decay has not been previously investigated.

3.3. Sensorial Analysis

Figure 3 represents variations in the sensory characteristics of table grape berries at 3 days of shelf life at 24 °C. Sensorial analysis was performed only in uncoated, coated and probiotic *L. plantarum* 11-A coated berries. As expected, artificial contamination of berries was found to impact the product's quality significantly negatively. In fact, among the parameters evaluated, those related to positive features, such as appearance, colour, freshness, firmness, and overall acceptance (Figure 3B, right side), were all ranked plenty lower than the limit of marketability for uncoated berries. At the same time, mould occurrence and off-odour (Figure 3B, left side), which were both related to fungal contamination, fermentations, and fruit tissue necrosis, were ranked at the top.

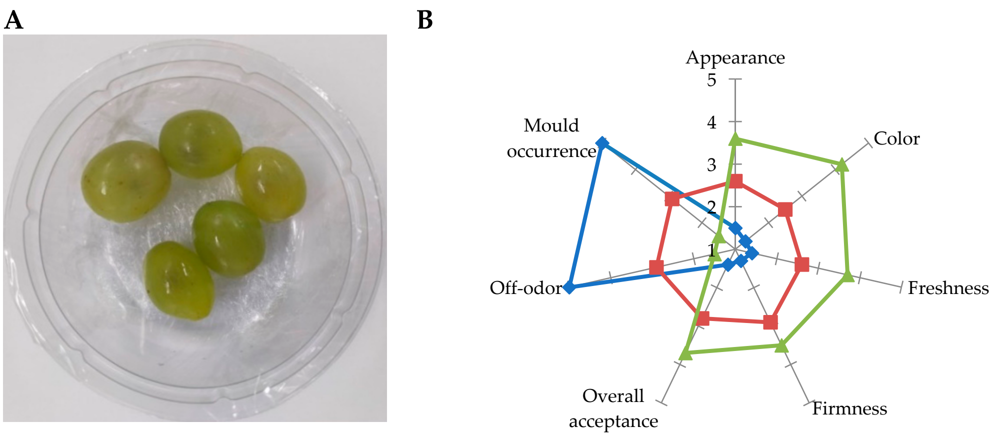

Figure 3. Example of probiotic table grapes cv. Italia portion (**A**). Sensorial evaluation of uncoated (blu), alginate coated (red) and probiotic *L. plantarum* 11-A coated table grapes cv. Italia berries artificially contaminated with *A. niger* CECT 2805 after 3 days of shelf-life at 24 °C (**B**).

Alginate-coated berries showed intermediate values of all the descriptors evaluated, with those related to positive features slightly below the limit of marketability. On the contrary, mould occurrence and off odour were ranked higher than the limit and considered unacceptable for marketing. Table grapes berries coated with *L. plantarum* 11-A showed the best performance, with positive feature values still higher than the limit of marketability and with low impact of mould occurrence and off odour even after fungal artificial contamination. For these reasons, table grape berries coated with the probiotic strain *L. plantarum* 11-A were considered to be of sufficient quality for marketing.

4. Conclusions

Fresh fruit and vegetables represent crucial factors in a balanced diet aimed at maintaining a healthy state. Due to their water and nutritional content, fruit and vegetables are very perishable foods, particularly due to the development of mould. In light of lifestyle changes, in order to maximize the presence of fruit and vegetables in the diet, it becomes crucial to optimize post-harvest management to improve the shelf life, quality and safety of ready-to-eat products. There is growing interest in postharvest applications of lactic acid bacteria for the production of ready-to-eat fruits and vegetables with selected LAB strains. In particular, these solutions enhance the antimicrobial and probiotic properties of selected strains to improve hygienic quality and functional characteristics.

Edible packaging solutions represent further useful solutions to improve the characteristics of the finished product appreciated by the market, protecting the product from a physical, chemical and biological point of view. This scientific work proposes a synergy between these two categories of innovations, using *L. plantarum*, alginate coating and table grapes as model factor for the experimental design. Alginate coating was used to improve the transfer of probiotic cells on table grape berry surfaces. With this strategy, probiotic cell transfer increased by about 2 Log CFU/g, and higher viability was maintained until the end of shelf-life. Significant differences in terms of decay prevention were also found in the alginate coating containing the probiotic strain *L. plantarum* 11-A. All the strains displayed the same behaviour after inclusion in alginate in terms of improvement of the number of cells. On the opposite, a strain-dependent effect was underlined for the properties linked to biocontrol properties after inclusion in the alginate matrix, suggesting that the application of antimicrobial microbes as bio-tools in edible packaging may represent an innovative criterion in the selection of lactobacilli to be exploited postharvest.

Author Contributions: Nicola De Simone: Writing – original draft, Methodology, Investigation, Data curation, Conceptualization. Angela Scauro: Methodology, Investigation, Data curation, Conceptualization. Danial Fatchurrahman: Investigation, Data curation. Pasquale Russo: Writing – review & editing, Supervision, Conceptualization. Vittorio Capozzi: Writing – review & editing, Supervision, Resources, Conceptualization. Mariagiovanna Fragasso: Resources, Conceptualization. Giuseppe Spano: Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization.

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