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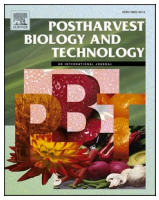

Probiotic *Lactiplantibacillus plantarum* strains showing anti-*Botrytis* activity: A food-grade approach to improve the overall quality of strawberry in post-harvest

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ABSTRACT

Strawberry is a highly perishable soft fruit susceptible to microbial contaminations, with *Botrytis cinerea* among the main spoilers in post-harvest. Lactic Acid Bacteria (LAB) are well-known food-grade bacteria, usually employed in food fermentation for their protechnological and probiotic properties. Moreover, LAB strains are also used as biocontrol agents for their ability to synthesise antimicrobial metabolites. However, applications of selected LAB to improve the overall quality in the fruit sector are still underexplored.

In this study probiotic *Lactiplantibacillus plantarum* strains showing anti-*Botrytis* activity were grown in strawberry juice (SJ). Probiotics were transferred to strawberries through dipping in fermented SJ, and the impact of the carrier matrix on their survival was evaluated in a simulated oro-gastrointestinal model. The best candidates were selected to investigate the postharvest quality of strawberries at different levels including functional (i.e., viability of probiotics at the end of the shelf life); safety (i.e., ability to control the growth of foodborne pathogenic bacteria); shelf life (i.e., biocontrol of *B. cinerea*); nutritional and sensory. The probiotic survival under simulated gastrointestinal conditions was up to 2.5 Log higher when strawberry was used as carrier. In co-inoculation assays on strawberries, *L. plantarum* 11 A and CB56 were able to reduce the growth of *Listeria monocytogenes* and *Escherichia coli* of about 1 Log after 7 days of cold storage. Moreover, live *L. plantarum* were able to control the growth of *B. cinerea* of about ten and five folds for strain 11 A and CB56, respectively, and a lower bioprotective effect was detected on strawberries dipped in the cell-free SJ fermented by *L. plantarum* 11 A. No significant effect was observed in terms of the main nutritional compounds, while improved descriptors related to the appearance of the fruit were observed. Therefore, this study allows us to elucidate the potential of selected LAB strains to improve the overall post-harvest quality of strawberries by using a thorough food-grade approach.

1. Introduction

Strawberry is a world-wide consumed soft fruit, highly appreciated as a source of bioactive compounds, including vitamins, health-promoting antioxidants, polyphenolic compounds, flavonoids, anthocyanins, and amino acids, as well for its organoleptic and sensorial quality (Giampieri et al., 2012). In 2021, the world's production reached more than 9 million tons, a duplicated value with respect to 2010 (FAOSTAT, 2024). However, strawberries are highly perishable and susceptible to safety concerns since the fruit grow close to the ground, are manually harvested, and are typically consumed raw (Yeargin et al., 2021). The main spoiler of strawberries is considered *Botrytis cinerea*, the

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grey mould's etiological agent, with very high relevance in terms of economic losses, estimated to reach 55 % and up to 89 % during harvest and post-harvest, respectively (Vanti et al., 2021). Strategies to extend the post-harvest life of strawberries include atmosphere modification (Colelli and Martelli, 1995), rapid cooling (Anderson et al., 2004), essential oils (Shehata et al., 2020), edible coatings (Gol et al., 2013), ultrasounds (Aday et al., 2013), pulsed light (Duarte-Molina et al., 2016), and the use of microbial antagonists (De Simone et al., 2021a). Bacterial strains, including *Bacillus halotolerans* and *Bacillus amyloliquefaciens*, as well as yeasts producing antifungal volatile organic compounds, have been successfully applied for the reduction of grey mould in strawberries (Maung et al., 2021; Ruiz-Moyano et al., 2020; Wang et al., 2021). However, though considered of biological class risk 1, most of these microorganisms should be submitted to a previous full-risk assessment by the regulatory authorities before their introduction into the food chain. In contrast, Lactic Acid Bacteria (LAB) have a long history of use in the food industry and/or as natural bio-preservation microorganisms, testified by the GRAS (Generally Recognized as Safe) and QPS (Qualified Presumption of Safety) status, by FDA (United States Food and Drug Administration) and EFSA (European Food Safety Authority), respectively. The employment of antagonistic LAB as biocontrol agents has been suggested as an attractive strategy to inhibit spoilage and/or pathogenic microorganisms on fruit, mainly due to competition for essential nutrients and as a result of the synthesis of different antimicrobial metabolites (Agriopoulou et al., 2020; Linares-Morales et al., 2018). In the fresh-cut sector, food-grade LAB strains have also been proposed for the biocontrol of foodborne pathogenic bacteria (i.e. *Salmonella* spp., *Escherichia coli* O157:H7, *Listeria monocytogenes*) (Iglesias et al., 2018; Siroli et al., 2016). For example, promising results to inhibit the growth of *L. monocytogenes* have been reported by using the nisin-producing *Lactococcus lactis* CBM21 or the probiotic *Lacticaseibacillus rhamnosus* GG on fresh-cut apples and pears, respectively (Iglesias et al., 2018; Siroli et al., 2016). To the best of our knowledge, only a few biocontrol candidates for controlling the grey mould in strawberries have been selected among LAB (Chen et al., 2020; Temmermans et al., 2023; Zamani-Zadeh et al., 2014).

On the other hand, some LAB strains show probiotic features, and their intake exerts different health benefits on the host. Probiotic fortification is a well-established approach to produce foods with functional properties (Siró et al., 2008). In particular, vegetables and fruit are promising alternatives to dairy products to deliver beneficial microbes, meeting the expectations of specific consumer targets (Min et al., 2019). Therefore, in recent years, many authors proposed different fruit as carriers for probiotic LAB (Alegre et al., 2011; Lillo-Pérez et al., 2021; Russo et al., 2015, 2014). The development of fruit with adequate probiotic cell concentrations at the time of consumption is a challenge in the functional food industry because intrinsic, processing and storage conditions severely affect bacterial viability (Fiocco et al., 2020). Moreover, supplementation with probiotic bacteria may affect the organoleptic and nutritional properties of the food throughout its shelf life (Alvarez et al., 2021). Therefore, several approaches have been explored to transfer probiotic/biocontrol cultures to the fruit, including dipping in solutions containing high concentration of live bacteria, encapsulation, or the employment of bioactive coatings (Khodaei and Hamidi-Esfahani, 2019; Misra et al., 2021). However, all of these methods foresee the LAB growth in expensive or not food-grade laboratory culture media.

In this study, previously characterised probiotic *Lactiplantibacillus plantarum* strains with broad antimicrobial activity (Rocchetti et al., 2023; De Simone et al., 2024b) have been proposed to enhance the overall quality of strawberry. In particular, the main goal of this study was to validate, at the laboratory scale, a comprehensive food-grade approach to obtain high cell viability of probiotic *L. plantarum* strains showing anti-*Botrytis* activity by fermentation of strawberry juice (SJ). The influence of the carrier matrix on probiotic survival has been evaluated in a simulated oro-gastrointestinal model. Finally, the impact of the addition of probiotics or their metabolites through dipping in fermented SJ or cell-free fermented SJ, respectively, on the overall quality (i.e., functional, nutritional, organoleptic, as well as safety and shelf life) of strawberries has been determined.

2. Materials and methods

2.1. Microbial strains and growth conditions

Five *L. plantarum* strains isolated from fruit and vegetables (Rocchetti et al., 2023) were routinely cultured in MRS broth (Oxoid, Basingstoke, United Kingdom) at 30 °C. Three foodborne pathogenic bacteria, namely *Escherichia coli* O157:H7 UFG77, methicillin-resistant *Staphylococcus aureus* UFG141 and *Listeria monocytogenes* CECT 4031, were inoculated from cryopreserved stock (1:1000 v/v) in Triptone Soya Broth (TSB) (Oxoid) and incubated at 37 °C for 24 h.

The filamentous fungus *Botrytis cinerea* CECT 20973 was propagated from cryopreserved culture on Potato Dextrose Agar (PDA) (Oxoid) plates at 24 °C for 5 d. 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, and stored at 4 °C for short-term uses. Fungal spores concentration was determined by plating serial dilution on PDA plates and adjusted to approximately 1×10^6 spores mL^{-1}.

2.2. Anti-Botrytis activity of probiotic L. plantarum strains

L. plantarum strains were grown on MRS broth for 18 h at 30 °C. The corresponding cell-free supernatants (CFS) were obtained by eliminating the pellet by centrifugation followed by filtration of the supernatant (0.22 μm pore-filter, VWR international, West Chester, PA, US). The anti-*Botrytis* activity of CFS was assayed by radial growth inhibition as previously described by De Simone and co-authors (2023). Briefly, each CFS was added to PDA plates at a concentration of 10 % (v/v). Control plates were supplemented with 10 % (v/v) of MRS broth. After solidification, 10 μL of a fungal suspension containing 1×10^4 spores mL^{-1} were spotted at the center of the plate. After 5 d of incubation at 25 °C, the antifungal ability was determined as percentage of hyphal radial growth compared to the control.

2.3. Growth of probiotic L. plantarum strains in strawberry juice

Strawberries were purchased from a local fruit retailer (Bovino, Italy) and immediately stored at 4 °C. Fruit with low-quality standards (e.g. non-homogeneous weights and sizes, bruising or other visual defects) were used to prepare strawberry juice (SJ). Fruit were washed in tap water and homogenised in a blender. To remove larger particles, the juice was centrifuged at 10,000 g for 20 min at 4 °C. Then, the supernatant was collected, and stabilised by thermal treatment at 60 °C for 60 min. *L. plantarum* strains were grown overnight in MRS broth until achieving a concentration of about 9 Log CFU mL^{-1}. Cultures were centrifuged (8,000 g for 1 min), washed twice and resuspended in sterile saline solution. In a preliminary assay, SJ was inoculated (1:1000 v/v) with each *L. plantarum* suspension. For further experiments, SJ was neutralised (pH 6.0 by using NaOH 1 M), and inoculated as above reported, by using different SJ concentrations (namely 25 %, 50 %, 75 %, and 100 % diluted in sterile water). Fermentation was performed at 30 °C for 24 h. Growth in MRS was used as control. Then, the pH of each sample was measured, and viable cells were enumerated by plate counting on MRS agar. The assay was carried out in triplicate.

2.4. Analysis of the carrier matrix on the survival of L. plantarum strains in a simulated oro-gastrointestinal model

Healthy fruit were sanitised by dipping for 1 minute in 0.01 % w/v sodium hypochlorite solution, rinsed twice with sterile demineralised

water, and dried under a laminar flow hood. Then, five strawberries were artificially contaminated by dipping for 1 min in 100 mL of each 24-h fermented SJ, and air-dried under laminar flow hood. Probiotic strawberries were digested using a three-compartment gastrointestinal model, as reported by Minekus et al., (2014), with slight modifications. The experiments were performed by using strawberries or saline solution containing the same amount of probiotic strains in order to assess any potential protective impact of the matrix during simulated oro-gastrointestinal tract. Briefly, probiotic strawberries or *L. plantarum* suspension containing approximately 2×10^7 CFU g^{-1} were diluted (1:2 v/v) with simulated salivary fluid supplemented with 150 mg L^{-1} of lysozyme at pH 6.0 and incubated for 5 min at 37 °C. Mastication was simulated by mincing in a pestle. Then, the gastric environment was simulated by diluting (1:2 v/v) with simulated gastric juice supplemented with 3 g L^{-1} of pepsin at pH 3.0 and incubating for 2 h at 37 °C. For the intestinal phase, the chilus was diluted (1:2 v/v) with simulated intestinal juice supplemented with 1 g L^{-1} of pancreatin at pH 6.5 and incubated for 2 h at 37 °C. All solutions used for the simulated digestion were prepared as Minekus et al. (2014) and sterilised by filtration. Probiotic viability in each phase was determined by plating on MRS agar. The assays were performed in triplicate.

2.5. Fruit biocontrol assay

Following the previous screening, two *L. plantarum* strains were selected for fruit biocontrol assay. Both strains were grown in 2 L of SJ for 24 h at 30 °C. After incubation, the SJ cultures were aliquoted in two batches, each containing 1 L of which one aliquot was used to obtain cell-free fermented SJ. Briefly, the juice was centrifuged (10,000 × g for 20 min at 4 °C) to eliminate most of the biomass, followed by filtration through 0.45 μm filter. Both cell-free SJ fermented and fermented SJ containing live bacteria were aliquoted (200 mL) in sterile plastic containers and contaminated or not (100:1 v/v) with foodborne pathogenic bacteria or spores of *B. cinerea*. Control samples was unfermented SJ. Fresh strawberries were prepared for the assay as above described and then 15 strawberries for each experimental condition were artificially contaminated by dipping for 1 min at 100 g in the corresponding solutions. After air-drying in laminar flowhood, samples were packed in polypropylene plastic bag (each containing 5 strawberries) under passive-modified atmosphere packaging conditions and stored at 4 °C for 7 d. Bacterial viability was checked by plate counting on MRS, CEC, PALCAM Listeria Selective Agar, and Mannitol Salt Agar for enumeration of *L. plantarum*, *E. coli*, *L. monocytogenes*, and *S. aureus*, respectively. Fungal contamination was evaluated after 3 days of storage at 24 °C, through image acquisition by using a vision computer system equipped with a digital color camera (EOS 00D, Canon, Melville, NY, USA) located vertically on a matte black background at a distance of 0.45 m. The images were processed using Matlab® R2021b (MathWorks Inc., Natick, MA, USA). The total area and the red area of each strawberry were calculated to get the percentage of red coverage as previuosly reported for strawberry (Corvino et al. 2023; Palumbo et al., 2022). For each strawberry, the percentage of infected area was calculated including the area covered by visible hyphal growth of fungi. Then, the infected area percentage was calculated with the following formula:

$$\text{Infected area (\%)} = \frac{\text{total area[n.of pixels]} - \text{red area[n.of pixels]}}{\text{total area[n.of pixels]}} \times 100$$

2.6. Analytical determination of the main nutritional compounds

The following analysis were performed at 0 and after 7 d of storage at 4 °C according to the procedures reported by Russo et al. (2014). All the determinations were carried out in triplicate.

2.6.1. Sugars and organic acids

Sugars and organic acids were extracted homogenising 15 g of fresh strawberry tissue with 15 mL of ultrapure water for 1 min. The homogenate was centrifuged at 10,000 g for 10 min at 5 °C. The supernatant was filtered with a C18 Sep-Pak cartridge and then with a 0.2 μm filter. Sugars and organic acids were identified using the method as described by Mena et al. (2011) and quantified by chromatographic comparison with analytical standards.

2.6.2. Total phenols and antioxidant capacity

Fruit extracts were obtained by homogenising 15 g of strawberries in an Ultra-turrax for 1 min with 20 mL of extraction medium, 2 mM NaF methanol:water solution (80:20 v/v). The homogenate was filtered through 2 layers of cheesecloth and then centrifuged at 5 °C at 10,000 g for 5 min. The supernatant was used to analyse total phenols and antioxidant activity. The content of total phenols was expressed as mg of gallic acid per 100 g of fresh weight. For antioxidant assay, 50 μL of the diluted sample were pipetted into 0.95 mL of DPPH solution to initiate the reaction. The absorbance was read at 515 nm after overnight incubation. Trolox was used as a standard and the antioxidant activity reported in mg of Trolox equivalents per 100 g of fresh weight.

2.6.3. Vitamin C

Vitamin C content was assessed homogenising 5 g of strawberry tissue for 1 min with 5 mL of methanol/water (5:95 v/v), plus citric acid (21 g L^{-1}), EDTA (0.5 g L^{-1}), and NaF (0.168 g L^{-1}). The homogenate was filtered, and the pH was adjusted to 2.2 by the addition of 6 M HCl. The homogenate was centrifuged at 10,000 g for 5 min, and the supernatant recovered, filtered through a C18 Sep-Pak cartridge (Waters, Milford, MA, USA) and then through a 0.2 μm cellulose acetate filter.

2.7. Sensorial analysis

A panel of ten trained panellists carried out the sensory evaluations of strawberries on the processing day and at the end of shelf-life. Before evaluations, panellists were trained in order to recognise and score the quality attributes. Translucency, dehydration, browning, flavour, firmness, juiciness, sweetness, acidity, off-flavour, off-odours, and colour were evaluated using a hedonic scale from 1 to 5, where 1 = not present/very low/not typical and 5 = very pronounced/very typical of fresh fruit.

2.8. Statistical analysis

The SAS statistical computer package was used to analyse the experimental data (SAS Institute, Cary, NC, USA). Analysis of variance (ANOVA) followed by Tukey's HSD post-hoc test was used to analyse data and determine statistically significant differences, with $p < 0.05$ as the minimal level of significance.

3. Results and discussion

3.1. Antifungal activity of probiotic L. plantarum strains against B. cinerea

Strawberries are susceptible to the colonisation of filamentous fungi during the post-harvest period, being *Botrytis cinerea* responsible for the grey mould (Vanti et al., 2021). Strains of *L. plantarum* are known to produce antifungal metabolites, and large cohorts have been screened for their anti-*Botrytis* activity in order to select potential biocontrol agents (De Simone et al., 2021b; Petkova et al., 2022). Therefore, in this work, five previously characterised probiotic *L. plantarum* strains with broad antimicrobial activity (Rocchetti et al., 2023) were tested for their antagonism against *B. cinerea* CECT 20973. Some significant differences in the degree of inhibition were detected as shown in Fig. 1. In particular, *L. plantarum* 11 A showed the highest level of anti-*Botrytis* activity, with about 65 % of mycelial growth inhibition. The other strains had levels of inhibition between 10 % and 15 % lower, being *L. plantarum*

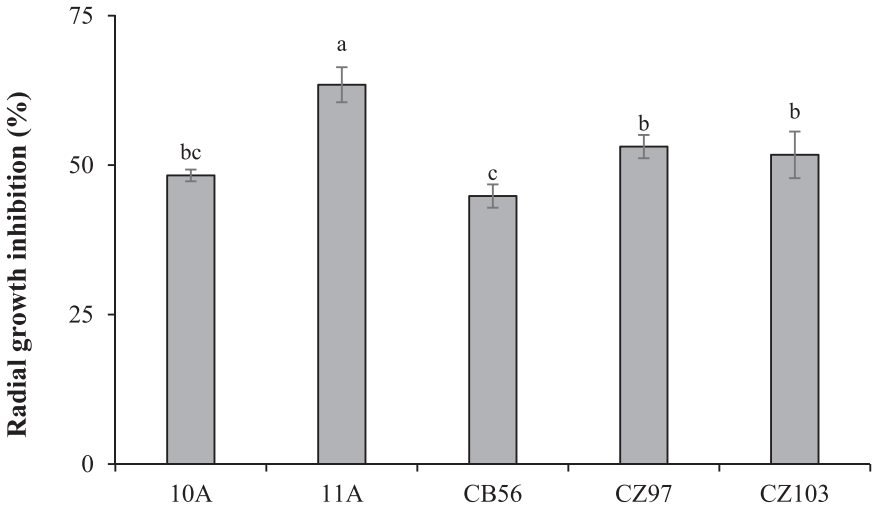

Fig. 1. Hyphal radial growth inhibition of *B. cinerea* CECT 20973 after 5 days of incubation at 24 °C on plates of PDA supplemented with 10 % of CFS obtained from the analysed *L. plantarum* strains. Values are the means and standard deviation of three biological replicates. Lowercase letters indicate significant differences as determined by ANOVA test ($p < 0.05$) followed by Tukey's test.

CB56 the strain showing the lowest anti-*Botrytis* potential (about 45 % of inhibition). Interestingly, the same strains were found to be able to inhhibit the growth of *Aspergillus niger* through the production of novel antifungal volatile organic compounds (De Simone et al., 2024a).

3.2. Optimisation of SJ as substrate for the growth of probiotic *L. plantarum* strains

Within the frame of a circular economy approach, we investigated if SJ obtained from the fermentation of healthy but not marketable strawberries (based on their colour, shape, and size) was a suitable substrate for the growth of *L. plantarum* strains. It is well known that large amounts of fresh fruit and vegetables are rejected for not meeting aesthetic standards, suggesting that their alternative employment is a challenge for a sustainable food industry (Siddiqui et al., 2023). In the last years, fruit wasting has attracted increasing attention as a valuable source for producing alternative fermented products (Salas-Millán et al., 2022). However, unlike other wastes from the food industry, such as cheese whey, that are excellent sources of nitrogen, the employment of fruit-based wastes for the biomass production of beneficial bacteria is poorly reported (Bernal-Castro et al., 2023; Kumar et al., 2022). SJ is rich in nutritional compounds (mainly sugars, phenolics, and flavonoids, but also micronutrients such as minerals and vitamins) required for microbial growth (Zhao et al., 2021). However, various factors could contribute to limiting the microbial growth in SJ, including acidic pH, high sugar or phenolic concentration, low nitrogen sources as well as the occurrence of antimicrobial compounds both of vegetable origin or chemical synthesis (e.g. residues of phytosanitary treatments) (Fiocco et al., 2020; Russo et al., 2019). In a preliminary assay, *L. plantarum* strains were inoculated in SJ whose pH was 3.3. However, after 24-h fermentation the viability was always lower than 5 Log CFU mL^{-1} (data not shown), a concentration unsuitable for the proposed biotechnological application. Accordingly, only few probiotic strains were able to tolerate the low pH of different juice matrices (Sheehan et al., 2007). Therefore, the pH of SJ was neutralised, and different concentrations of SJ were tested. The viability and corresponding pH values at the end of the fermentation were compared with a commercial culture medium (MRS) commonly used for the growth of Lactobacilli, and the results are reported in Table 1. After fermentation the concentration of viable cells increased until about 9 Log CFU mL^{-1}, a level slightly lower than what obtained in MRS, thus confirming the suitability of SJ juice as a food-grade substrate to support the growth of *L. plantarum* strains. As expected, the viability of the strains gradually decreased reducing the concentration of SJ, ranging until approximately 7 Log CFU mL^{-1} when the substrate was diluted at 25 %, probably due to a lower concentration of carbon sources and other nutritional compounds. SJ has been proposed as an ingredient for the production of functional fermented dairy and non-dairy beverages due to its health benefits and good acceptability by consumers (Balthazar et al., 2019; Corona et al., 2016; Paredes et al., 2022). However, only a few studies evaluated the capability of LAB strains to ferment pure SJ (Zhao et al., 2021). According to our results, it has been reported that *Lactobacillus acidophilus* and *L. plantarum* were able to achieve maximum levels of more than 9 Log CFU mL^{-1} after 12 h of SJ fermentation (Chen et al., 2023), while *Levilactobacillus brevis* CRL 2013 grew at a final concentration of about 7.3 Log CFU mL^{-1} (Cataldo et al., 2020). In contrast to our results, Cataldo et al., (2020) did not find significant differences in cell viability between juices with neutralised and non-adjusted initial pH, but reported a

Table 1
Viability and pH values of *L. plantarum* strains after 24 h of fermentation at 30 °C in SJ at pH 6.0 diluted at different concentrations (i.e., 100 %, 75 %, 50 %, and 25 %) or MRS. Values are the means and standard deviation of three biological replicates. Data are the means ± SD of three replicates. Capital letters indicate significant differences between SJ at different concentration and MRS at all the experimental conditions. Lowercase letters indicate significant differences among the strains inside each experimental condition. Values with different letters are significantly different according to one-way ANOVA test ($p < 0.05$) followed by Tukey's multiple comparison test.

Strain	100 % SJ		75 % SJ		50 % SJ		25 % SJ$^{(ns)}$		MRS$^{(ns)}$	
	LogCFU mL^{-1}	pH	LogCFU mL^{-1}	pH	LogCFU mL^{-1}	pH	LogCFU mL^{-1}	pH	LogCFU mL^{-1}	pH
11 A	8.94 ± 0.02^{Bab}	4.80	8.62 ± 0.05^{Cab}	4.83	7.85 ± 0.03^{Dabc}	4.89	7.01 ± 0.04^{E}	5.03	9.51 ± 0.16^{A}	3.75
CB56	9.18 ± 0.20^{Ba}	4.78	8.68 ± 0.04^{Cab}	4.80	7.89 ± 0.02^{Dab}	4.77	7.16 ± 0.17^{E}	4.95	9.59 ± 0.14^{A}	3.86
10 A	8.99 ± 0.08^{Bab}	4.94	8.71 ± 0.03^{Ba}	4.95	7.94 ± 0.06^{Ca}	5.01	7.22 ± 0.25^{D}	5.04	9.66 ± 0.06^{A}	3.91
CZ97	8.95 ± 0.02^{Bab}	4.99	8.54 ± 0.06^{Cb}	5.01	7.80 ± 0.08^{Dbc}	5.05	7.34 ± 0.12^{E}	5.12	9.65 ± 0.04^{A}	3.78
CZ103	8.91 ± 0.03^{Bb}	5.02	8.75 ± 0.08^{Ca}	5.02	7.74 ± 0.02^{Dc}	5.05	7.26 ± 0.08^{E}	5.08	9.63 ± 0.03^{A}	3.89

positive impact of the addition of yeast extract or tryptein (Cataldo et al., 2020). Recently, it has been reported that adaptation of *L. plantarum* with citric acid significantly improved subsequent survival in highly acidic fruit juices (Srisukchayakul et al., 2018). Thus, these preliminary results indicated that fermentation conditions of SJ could be further optimised in order to obtain a higher cell density.

3.3. Effect of the carrier matrix on the survival of probiotic L. plantarum strains in a simulated oro-gastrointestinal model

Our results showed that after dipping, strawberries contains about 7 Log CFU g^{-1} of *L. plantarum* strains (Fig. 2A), a concentration consistent with probiotic food products (Binda et al., 2020). As previously reported for other fresh-cut fruit, a dipping step is a faster and cheaper approach to transfer probiotic LAB strains to the fruit surface (De Simone et al., 2023; Russo et al., 2015, 2014).

The protective effect of the fruit matrix was evaluated by comparing the LAB viability during simulated digestion of probiotic strawberries (Fig. 2A) or probiotic suspensions in saline solution (Fig. 2B). As shown in Fig. 2, the probiotic strains were able to withstand the typical condition of the oral phase in both assays (i.e., suspension and fruit matrix), showing a minimal reduction in viability. In contrast, under simulated gastric conditions, a higher reduction of viability of both strains was detected when probiotics were administered through saline suspension. In particular, *L. plantarum* 11 A showed higher survival with more than 4.45 Log CFU g^{-1} of viable cells, followed by *L. plantarum* and CZ97 with 4.03 and 3.99 Log CFU g^{-1} (Fig. 2B). In contrast, under the same conditions all the strains maintained high viability (about 7 Log CFU g^{-1}) when administered through strawberry (Fig. 2A). A further reduction of viable cells was found when probiotics reached the simulated intestinal environment. In saline suspension, *L. plantarum* CB56 and *L. plantarum* 11 A showed higher viability, corresponding to 3.95 and 3.49 Log CFU g^{-1}, respectively, while in strawberry a survival of about 5.5 Log CFU g^{-1} was observed for both strains. At the end of the digestion process the probiotic viability was between 4.90 and 5.53 Log CFU g^{-1} when the strains were delivered by the fruit. On the contrary, when probiotics were administered through the saline solution, the survival was between 2.48 and 3.95 Log CFU g^{-1}. The viability was generally between 1 and

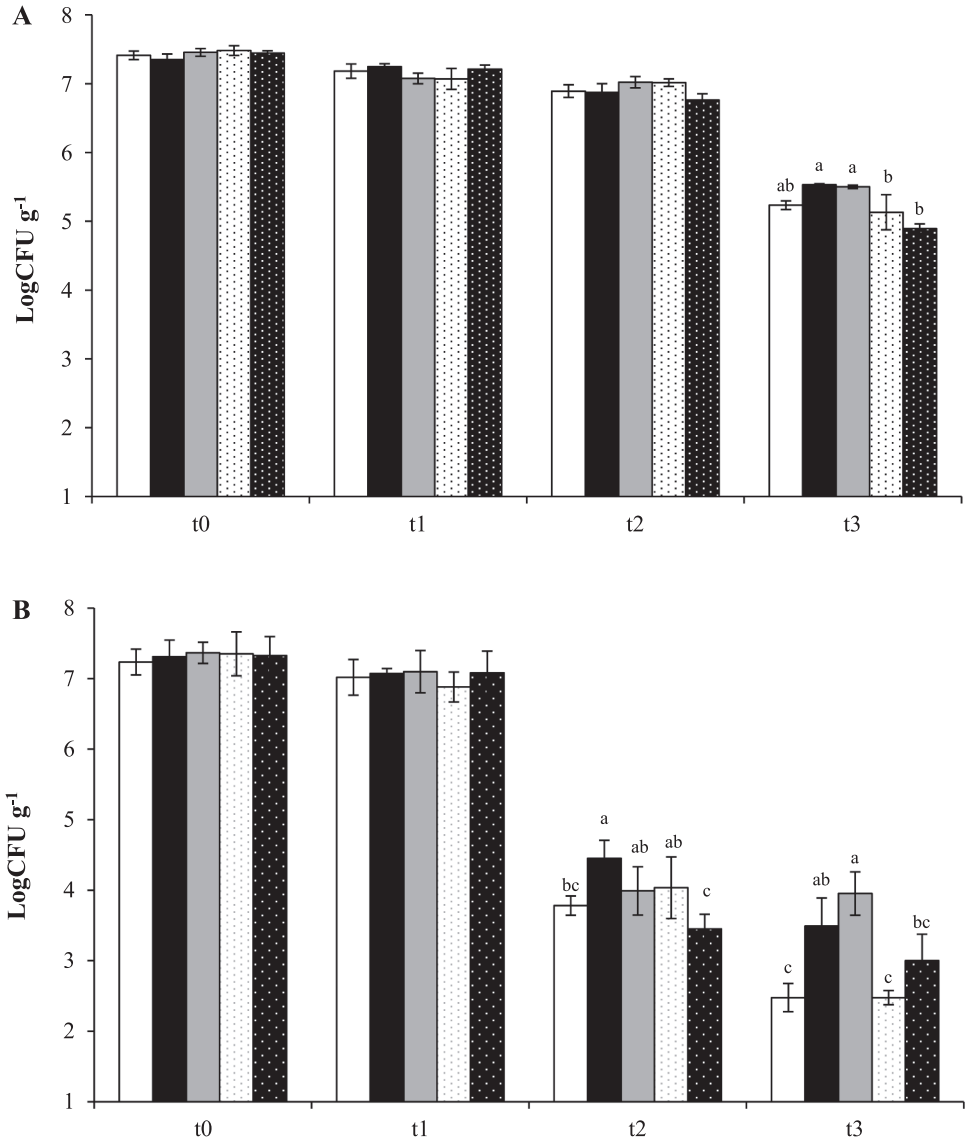

Fig. 2. Relative survival in different stages of simulated digestion of *L. plantarum* strains inoculated on strawberry (**A**) or saline solution (**B**). t0: initial concentration; t1: oral phase; t2: gastric phase; t3: intestinal phase. *L. plantarum* 10 A (white bars); *L. plantarum* 11 A (black bars); *L. plantarum* CB56 (grey bars); *L. plantarum* CZ97 (dotted bars on white background); *L. plantarum* CZ103 (dotted bars on black background). Data are reported as mean ± standard deviation of three different replicates. Lowercase letters indicate significant differences as determined by one-way ANOVA test ($p < 0.05$) followed by Tukey's test.

2.5 Log higher when strawberry was employed as a carrier. These results suggested that strawberries can help probiotic bacteria to face stress conditions, and enhancing their survival during oro-gastrointestinal transit as previously reported for other food matrices (Bove et al., 2013; Bustos et al., 2023). Therefore, based on the the obtained results, *L. plantarum* 11 A and CB56 were selected for further applications.

3.4. Impact of probiotic LAB on the overall quality and safety of strawberries

3.4.1. Impact on functional quality

Probiotic foods should ensure a concentration of at least 7 Log CFU g^{-1} of viable bacteria (Terpou et al., 2019). This value was consistent with what determined for both probiotic strains after dipping, and a similar concentration was found at the end of the shelf life (Table 2A). Moreover, after 7 days of storage the viability of *L. plantarum* 11 A and CB56 did not undergo significant changes even in co-contamination assays with pathogenic bacteria, indicating that their occurrence did not negatively affect the persistence of probiotc strains on the strawberry surface, as previously reported for apples, pineapples, cantaloupe, and pears (Alegre et al., 2011; Iglesias et al., 2018; Russo et al., 2015, 2014). In contrast, berries such as table grapes showed a lower rate of contamination after dipping, and alternative solutions such as edible coating has been proposed to transfer the same probiotic strains to the fruit surface (De Simone et al., 2024b). However, considering that a standard serving of strawberries correspond to about 150 g of product, the resulting amount of probiotic ingested could be estimated to approximately 5 $\times 10^9$ viable cells, a level suitable to the recommended functional daily dose according to the international consensus (Marco et al., 2021).

3.4.2. Impact on food safety

Three main foodborne bacterial pathogens (i.e., enterohemorrhagic *E. coli*, *L. monocytogenes*, and *S. aureus*) have often been associated with fresh produce outbreaks (Carstens et al., 2019; Ortiz-Solà et al., 2020). The development of innovative strategies has been explored to reduce the microbial risk and improve the overall quality of strawberries (Lafarga et al., 2019). Some LAB strains can act as biocontrol agents either directly through competition phenomena or indirectly by the synthesis of antimicrobial compounds (Russo et al., 2017). Therefore, the antagonistic effect of live probiotics or metabolites synthesised during the juice fermentation against these pathogens was determined *in vivo* on artificially contaminated strawberries, and results are summarised in Table 2B. Despite being inoculated at the same concentration, the pathogens showed a different capability to adhere to the fruit surface, with *E. coli* UFG77 showing the best performance, namely 1 Log higher than that observed for *L. monocytogenes* CECT 4031. In contrast, *S. aureus* UFG141 was detected at a concentration of about 3 Log CFU g^{-1} after dipping of the strawberry, a value that was slightly further reduced in co-contamination assay. These results suggested a limited adhesion capability that could be attributable to different strain or species-specific features as well as to unknown interactions occurring in complex food matrices. After 7 days of cold storage, the concentration of *L. monocytogenes* CECT 4031 and *E. coli* UFG77 was significantly reduced of about 1 Log when fruit were inoculated with live probiotics, and, at lower extent, some differences were also detected among strawberries dipped in the cell-free fermented SJ and the corresponding control. *S. aureus* UFG141 was never detected after 7 days of storage (less than 2 Log CFU g^{-1}) indicating a lower persistance on the fruit.This evidence indicated that selected probiotics are a potential strategy to control the growth of foodborne pathogenic bacteria in strawberries, corroborating what previously observed for other fresh-cut fruit (Iglesias et al., 2018, 2017a, 2017b; Russo et al., 2015, 2014).

Table 2
Viability of probiotic (**A**) and pathogenic (**B**) strains on artificially contaminated strawberries. Viability was determined after inoculation (t0) and 7 d of storage at 4 °C in passive modified atmosphere (t7). Values are the means and standard deviation of three biological replicates. Lowercase letters indicate significant differences as determined by ANOVA test ($p < 0.05$) followed by Tukey's test.

(A)	Viable Probiotic Count (Log CFU g^{-1})			
	L. plantarum 11 A		*L. plantarum* CB56	
	$t0^{(n.s.)}$	$t7^{(n.s.)}$	$t0^{(ns)}$	$t7^{(n.s.)}$
LAB	6.25 ± 0.16	6.39 ± 0.21	6.49 ± 0.15	6.52 ± 0.13
LAB+EC	6.08 ± 0.15	6.42 ± 0.10	6.74 ± 0.16	6.61 ± 0.12
LAB+SA	6.76 ± 0.01	6.44 ± 0.15	6.80 ± 0.10	6.29 ± 0.22
LAB+LM	6.58 ± 0.12	6.32 ± 0.21	6.99 ± 0.21	6.40 ± 0.13

(B)	Viable Pathogens Count (Log CFU g^{-1})					
	E. coli UFG77		*L. monocytogenes* CECT 4031		*S. aureus* UFG141	
	$t0^{(n.s.)}$	t7	$t0^{(n.s.)}$	t7	t0	t7
Pat	4.92 ± 0.08	4.81 ± 0.33^{a}	3.99 ± 0.41	3.00 ± 0.14^{ab}	3.16 ± 0.36^{a}	n. d.
Pat+11 A	4.80 ± 0.45	4.01 ± 0.27^{ab}	3.77 ± 0.65	2.30 ± 0.14^{c}	2.72 ± 1.01^{ab}	n. d.
Pat+CB56	4.79 ± 0.50	3.95 ± 0.19^{b}	3.96 ± 0.48	2.59 ± 0.16^{bc}	2.35 ± 0.49^{b}	n. d.
Pat+11A-CFS	5.12 ± 0.41	4.43 ± 0.31^{ab}	3.85 ± 0.85	2.15 ± 0.21^{c}	2.00 ± 0.27^{b}	n. d.
Pat+CB56-CFS	4.79 ± 0.44	4.43 ± 0.36^{ab}	3.82 ± 0.81	3.09 ± 0.18^{a}	2.90 ± 0.41^{ab}	n. d.

n.d. not detected
n.s. not significant

3.4.3. Impact on shelf life

As shown in Fig. 3, control strawberries were vulnerable to phenomena of microbial spoilage attributable to the presence of epiphytic microflora. As expected, when the fruit were artificially contaminated, *B. cinerea* covered almost all of their surface (about 93 %) after 3 days of storage at 24 °C. Interestingly, when strawberries were dipped in fermented SJ containing live probiotics, mould growth was lower (about 11 % and 20 % for *L. plantarum* 11 A and CB56, respectively). A bioprotective effect, although of lesser efficacy, was also detected on strawberries dipped in the cell-free SJ fermented by *L. plantarum* 11 A (approximately 30 % of surface covered by the mycelium), while treatment with cell-free SJ fermented by *L. plantarum* CB56 resulted in a contamination similar to the control, according to the lower anti-*Botrytis* potential of this strain as detected in *in vitro* assay. These results suggested that competition phenomena for substrates or nutritional factors were the main responsible for the observed antagonism or that specific antimicrobial compounds could be synthesised only in co-culture with the fungal strain (De Simone et al., 2024a). Nonetheless, the production of secondary metabolites during strawberry fermentation conferred a protective action, though at a lower extent. Similar results have been described in fresh-cut kiwifruit, suggesting the potential of different *L. plantarum* strains for the biocontrol of *B. cinerea* on different fruit matrices (De Simone et al., 2021b). Moreover, the same strains were able to contrast in a strain depending way the growth of *A. niger* in edible alginate coated table grapes (De Simone et al., 2024b)., The application of *L. plantarum* A7 with thyme and cumin essential oils has been reported as a potential biocontrol tool in post-harvest stage of strawberries (Zamani-Zadeh et al., 2014). In a recent study, *L. plantarum* AMBP214 dispersed to strawberry flowers via bumblebees showed a protective effect against *B. cinerea* in a greenhouse trial (Temmermans et al., 2023), suggesting its potential as a biocontrol agent also for pre-harvest biotechnological applications.

3.4.4. Impact on nutritional and sensory quality

To verify that the addition of the *L. plantarum* strains had no undesirable effects on the nutritional and organoleptic quality of the fruit, the main chemical parameters were determined in fermented or not fermented SJ, as well as in the fruit after dipping and 7 days of cold storage

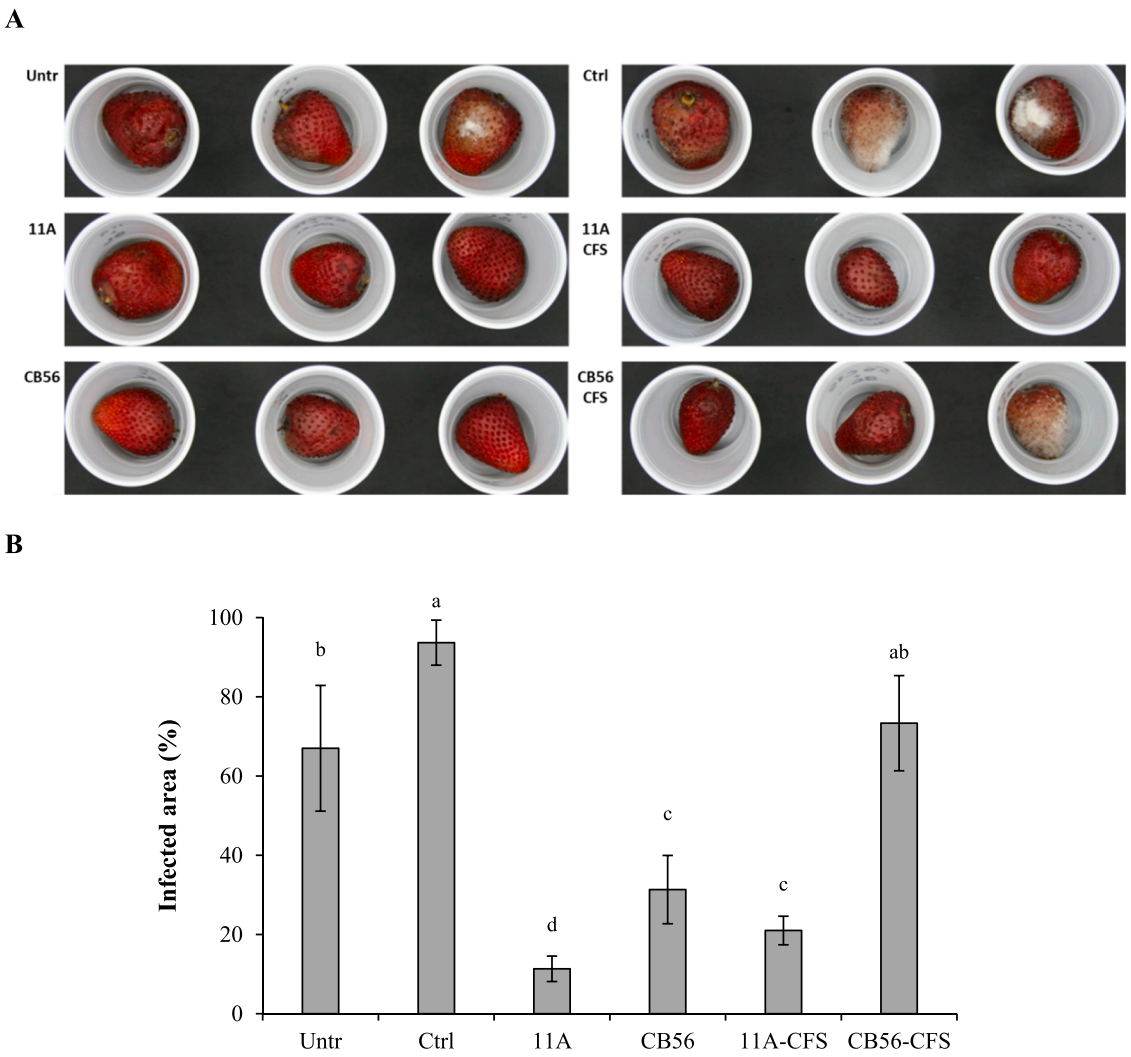

Fig. 3. Strawberries artificially contaminated with *B. cinerea* CECT 20973 (**A**). Strawberries not (Untr), or artificially contaminated with *B. cinerea* (Ctrl) and dipped in SJ fermented with *L. plantarum* 11 A (11 A) or *L. plantarum* CB56 (CB56) or cell-free SJ fermented with *L. plantarum* 11 A (11A-CFSJ) or *L. plantarum* CB56 (CB56-CFSJ) after 3 days of storage at 24 °C. Percentages of infected area in strawberries artificially contaminated with *B. cinerea* CECT 20973 after 3 days of storage at 24 °C as determined by image analysis. Data are the means ± SD of five replicates. Values with different letters are significantly different according to one-way ANOVA test ($p < 0.05$) followed by Tukey's multiple comparison test (**B**).

(Supplementary Table S1). As expected, fermentation of SJ by the *L. plantarum* strains determined a reduction of both glucose and fructose and a corresponding increase of organic acids. Interestingly, fermentation with *L. plantarum* CB56 enhanced the vitamin C content, while both strains were responsible for a more than five-fold increase in the total phenolic content. These results were related to an improved antioxidant capacity of both fermented SJ, being this value higher when fermentation was performed by *L. plantarum* 11 A. Several authors reported that fermentation by LAB resulted in an enhanced content of different bioactive compounds. In particular, an increase in vitamin C and phenolic was observed after lactic acid fermentation of beetroot juice, white cabbage, okra seeds, pomegranate juice, and kiwifruit pulp probably due to a strain-specific capability to reduce the oxidation of this vitamin (Adetuyi and Ibrahim, 2014; Chen et al., 2022; Kusznierewicz et al., 2008; Rakin et al., 2007; Valero-Cases et al., 2017). However, only some minimal differences were observed in strawberries dipped in SJ or fermented SJ indicating that including this technological step doesn't affect fruit nutritional quality. Furthermore, all the monitored parameters were almost stable during the storage time suggesting that also strawberries carrying out a high concentration of probiotics were not subjected to unwanted fermentation. These results are consistent with what was already reported for fresh-cut pineapple, cantaloupe, and pear (Iglesias et al., 2018; Russo et al., 2015, 2014).

The sensory analysis indicated a preference for fruit inoculated with probiotics, especially for those descriptors related to appearance (e.g. translucency, colour, dehydration), with less marked differences in the production of extraneous odours (Fig. 4). These data seem to be linked to what was observed by comparing probiotic fruit with control samples (Fig. 3A). On the other hand, some differences seem to be found between the two strains of *L. plantarum,* which could be due to strain-specific metabolic capabilities. In a previous study, it was reported that *L. plantarum* B2 impaired some sensorial attributes of fresh-cut cantaloupes but had no negative implications in pineapples (Russo et al., 2015, 2014), suggesting that the sensorial quality of low-acidic fruit could be compromised more easily by the metabolic activity of probiotic LAB. On the other hand, volatile compounds detected in probiotic pear wedges seemed to improve the flavour (Iglesias et al., 2018). Therefore, the impact of the addition of probiotic microorganisms on the organoleptic characteristics of strawberries could be further investigated by means of analytical techniques aimed at identifying the volatome corresponding to the different treatments (Romano et al., 2015).

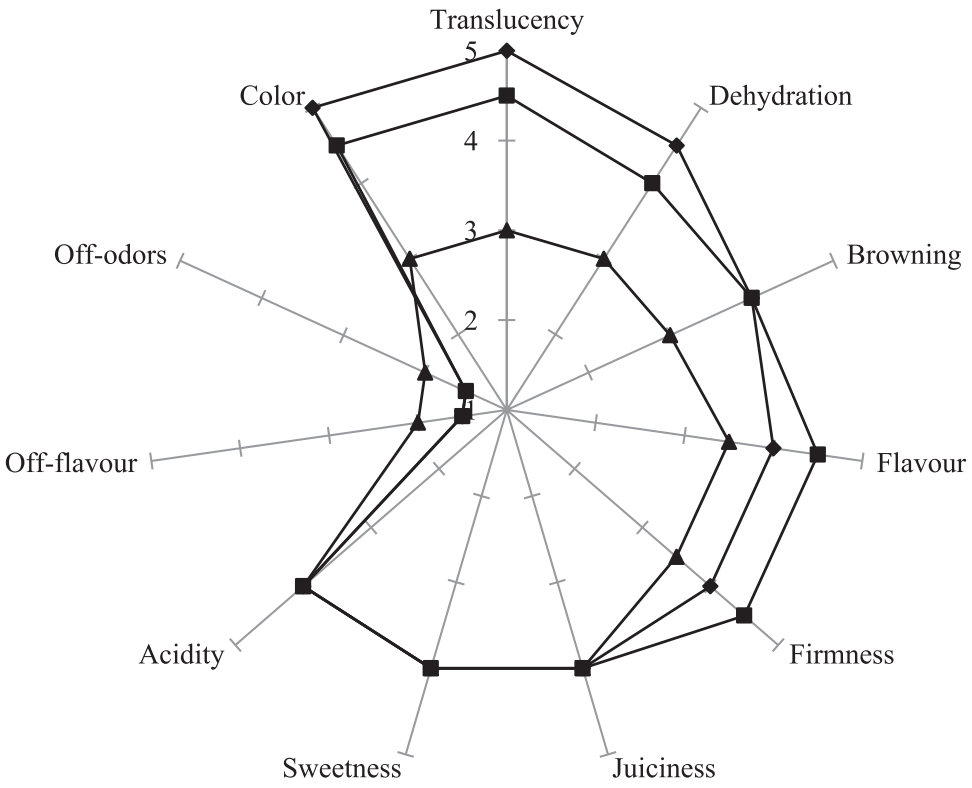

Fig. 4. Sensory properties of strawberries dipped in unfermented SJ (triangle), or SJ fermented 24 h with *L. plantarum* 11 A (diamond) or *L. plantarum* CB56 (square) and stored for 7 d at 4 °C. Values are the mean of three replicates for each sample and are expressed using a hedonic scale from 1 to 5 (1 = not present/very low/atypical and 5 =very pronounced/very typical).

4. Conclusions

In conclusion, this study aimed to investigate a comprehensive food-grade approach to obtain high-value microbial biomass by fermentation of non-marketable strawberries. This strategy could provide several technological advantages mainly including *i)* the use of low-cost and/or the reuse of waste material as substrate for the growth of beneficial microbes; *ii)* an easy and fast way to transfer probiotic/bioprotective LAB to the fruit surface; *iii)* the possibility to deliver only antimicrobial compounds by using cell-free fermented juice; *iv)* the suitability of bacteria pre-adapted to the food matrix; *v)* the absence of foreign compounds, as for the employment of essential oils, negatively impacting on the sensory quality. Therefore, this study meets critical innovation solutions (e.g., sustainable reduction of post-agricultural losses, increasing the value of waste or by-products, food safety, and consumer health) in post-harvest handling by proposing strawberries as a fruit model.

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CRediT authorship contribution statement

Pasquale Russo: Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization. **Nicola De Simone:** Writing – original draft, Visualization, Investigation, Formal analysis, Data curation. **Angela Scauro:** Investigation, Formal analysis, Data curation. **Danial Fatchurrahman:** Validation, Investigation, Formal analysis. **Giuseppe Spano:** Writing – review & editing, Supervision. **Mariagiovanna Fragasso:** Visualization, Data curation. **Maria Luisa Amodio:** Supervision, Resources, Methodology. **Vittorio Capozzi:** Writing – review & editing. **Giancarlo Colelli:** Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.postharvbio.2024.113125.

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