Article

Combined Microbiological Tools to Assess the Suitability of Lactic Acid Bacteria Cell-Free Supernatant as a Bio-Preservative in Ready-to-Eat Orange Against Wild *Staphylococcus aureus* and *Bacillus cereus* Isolates

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Abstract

The increased consumption of ready-to-eat fruits highlights the need for better control of microbial growth during their shelf life. Among bacteria, *Staphylococcus aureus* and *Bacillus cereus* are proposed as target species for testing alternative preservative methods. This study aimed to evaluate the antimicrobial effect of the cell-free supernatant (CFS) from LAB strains previously isolated from ready-to-eat fruits, used as a mixed solution, against both reference and native *S. aureus* and *B. cereus*, which were isolated from commercial ready-to-eat fruits. A specific challenge test was conducted on minimally processed orange slices, assessing the effect of CFS on the intentionally inoculated target bacteria using a culturing and quantitative PCR (qPCR) approach. Microbiological counts varied widely among samples, indicating an initial microbiota below legislative limits, mainly comprising total mesophilic and psychrophilic bacteria, which increased significantly after 8 days of storage. Additionally, our results demonstrated the food matrix's capacity to support the growth of both target species, with the tested CFS mainly effective in reducing the growth of reference strains. The results of the physicochemical analyses showed that during refrigerated storage, the orange slices underwent changes in pH, color, and texture, mostly in *S. aureus* strain-inoculated samples, negatively affecting texture at mid-storage time. The study also underscored the importance of combining plate counting with qPCR methods to detect *B. cereus*, as it can be risky even at low levels.

Keywords: cell-free supernatant; ready-to-eat fresh fruits; qPCR; *Staphylococcus aureus*; *Bacillus cereus*

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1. Introduction

Despite recent efforts to implement food safety, foodborne pathogens continue to pose a risk to consumer health. Recent cases of serious foodborne illnesses registered in Italy during the summer of 2025 underscore the importance of microbiological traits of vegetable products, as the demand for fresh-cut and ready-to-eat fruits (RTEFs) has enormously increased. The growing public interest in food safety has motivated the food industry

to pursue innovative processes and technological advancements in manufacturing [1] to achieve high microbiological quality levels during the entire shelf-life [2,3]. While they are nutritious, RTEFs offer medical and low-calorie benefits, along with convenience and immediate usability [2,4–6]. These products, characterized by high water content and damaged tissues, are susceptible to spoilage and can support the growth of microbial contaminants, including pathogenic microorganisms [2,5,7]. The production system, which includes washing, peeling, slicing, chopping, or shredding, followed by bagging or packaging and refrigeration, can lead to faster physiological deterioration and biochemical changes. This affects the durability and safety of the products, posing risks to public health, as they are consumed without further treatment [8]. The native microbial populations of RTEFs and RTE vegetables can vary significantly, comprising viruses, bacteria, yeasts, and molds. Among pathogens, *Listeria monocytogenes*, *Escherichia coli*, *Staphylococcus aureus*, *Bacillus cereus*, and *Salmonella* spp. have been the primary causative species associated with diseases related to the consumption of such types of food [1,3,9]. Among the various proposed strategies aimed at replacing traditional technological processes or preservative methods, biopreservation has gained increasing interest in maintaining higher nutritional and sensory quality levels. Biopreservation is based on naturally occurring or selected microorganisms and/or their metabolic products able to inhibit the growth of undesirable microorganisms [2,10]. Among the microbial-based preservation, the use of inactivated microbial cells or cell components, with or without metabolites, represents an evaluable alternative to live microorganisms due to the minimal risk of compromising sensory properties or nutrient levels [11,12]. Antimicrobial metabolites from multi-species lactic acid bacteria (LAB) consortia, containing organic acids (lactic, citric, and acetic acid), hydrogen peroxide, CO_2, diacetyl, ethanol, acetaldehyde, acetoin, ammonia, bacteriocins, and bacteriocin-like inhibitory substances, are suitable as preservatives for RTE food [5,11]. It is significant that, when combined with modified atmosphere packaging (MAP) or vacuum packaging (VP) and refrigerated storage, biopreservation achieves a cumulative effect in countering microbial growth [13]. Notably, both *S. aureus* and *B. cereus* are considered the main food safety hazards, causing food intoxication and infectious diseases. However, while mandatory microbiological criteria include the evaluation of *S. aureus* presence, currently, no legislation requires systematic screening of food items for *B. cereus* contamination, except for dried infant formula [14]. Moreover, several cases of foodborne disease due to *B. cereus* have been linked to bacterial concentrations of less than or equal to 10^3 CFU/g, which is particularly challenging to detect using cultivation-dependent methodologies [15].

Thus, the objectives of this study were to (i) isolate and characterize, using phenotypic and molecular approaches, wild RTEF bacteria belonging to *S. aureus* and *B. cereus* species; (ii) evaluate the in vitro antimicrobial activity of CFSs from autochthonous LAB against the target microorganisms; and (iii) assess, through a specific challenge test on minimally processed orange slices, the potential inhibition effects of the CFS against the intentionally inoculated target bacteria, during refrigerated storage (up to 14 days) using culturing and quantitative PCR (qPCR) approaches. Orange fruits were chosen for their global economic importance and as they represent an ideal matrix for harboring a limited native microbiota.

2. Materials and Methods

2.1. Experimental Plan

This study evaluates the antimicrobial effect of CFSs from autochthonous LAB, previously isolated from fruits [11], against *S. aureus* and *B. cereus* isolated from RTEFs. In detail, the LAB strains, namely AS1, AS2, and MEL1 and MAC1, belonging to *Lactiplantibacillus plantarum* and *Leuconostoc mesenteroides*, respectively, previously characterized for safety and technological

properties [11], were used as mix CFS against *S. aureus* and *B. cereus*, both constituted from indigenous and reference strains (*S. aureus* DSM 2569 and *B. cereus* DSM 626).

The indigenous *S. aureus* and *B. cereus* were isolated from commercial RTEF products and inoculated both in single and mixed cultures, as indigenous and reference strains (*S. aureus* DSM 2569 and *B. cereus* DSM 626), in a challenge test, structured according to the ISO 20976-1:2019 standard [16], on RTE orange slices. The same samples were treated with CFS from the autochthonous LAB and monitored up to 14 days under refrigerated conditions at 4 °C.

2.2. Isolation of Wild Target Bacteria (S. aureus and B. cereus) from Commercial RTEF

Different RTEFs, including orange slices, cantaloupe, fruit salad, and pineapple, with two packages per fruit type, purchased from local markets, were tested using routine cultural detection methods according to the microbiological criteria in EC Regulation No 2073/2005. Specifically, 25 g of each RTEF product was weighed into a stomacher bag with a filter (Interscience, Paris, France) and homogenized at a 1:10 diluent ratio (0.9% NaCl). Decimal dilutions of the homogenate were prepared using the same diluent. Then, 0.1 mL of the appropriate dilutions was surface plated on Rapid *E. coli* 2 (Biorad, Laboratories, Inc., Milan, Italy), Chromatic™ *E. coli* O157, and Mannitol Salt Agar (MSA) media, for *Escherichia coli*, *E. coli* STEC, and coagulase-positive staphylococci enumerations, respectively. Plates were aerobically incubated at 37 °C for 24–48 h. Moreover, a two-stage enrichment method for *Listeria monocytogenes* detection (International Organization for Standardization: ISO 11290-1:2017) [17] and *Salmonella* spp. detection (ISO 6579-1:2017) [18] was used. In addition, the Count Agar (PCA), aerobically incubated at 30 °C for 48–72 h and at 8 °C for one week, was used for total mesophilic and psychrophilic aerobic bacteria count, respectively. *Bacillus cereus* agar base (BCA), aerobically incubated at 30 °C for 24 h, was used for the enumeration of *B. cereus*. All media were purchased from Liofilchem srl (Roseto degli Abruzzi, Italy). Analyses were conducted in duplicate.

2.3. Identification of Presumptive S. aureus and B. cereus Isolates from RTEF

From all plates of MSA, BCA, and PCA, incubated at 30 °C, up to 20 typical colonies per sample were randomly selected and purified through three successive streaking steps on agar plates of the same medium. Based on phenotypic characteristics (Gram staining, microscopic observation, catalase test, hemolysis test, coagulase test, ability to ferment mannitol, and lecithinase production), the isolates were gathered into two main groups: presumptive *S. aureus* or *B. cereus*. For the identification at the species level, a qPCR analysis was performed. To confirm the *S. aureus* affiliation species, the amplification of the *spa* gene was performed, as previously reported by Russo and co-workers [19] and following the EU Reference Laboratory for antimicrobial resistance (EURL-AR) [20]. For the identification of *B. cereus*, the qPCR analysis was set up targeting a fragment of the *mot*B gene, a component of the flagellar motor of the *B. cereus* group.

In detail, the primer set (BCFomp2/BCRomp2), properly designed on the 186 bp *mot*B target gene (synthesized by Eurofins MWG Operon, Martinsried, Germany), was used in a 25 µL reaction mixture consisting of 2 µL DNA and 12.5 µL 2X PCR Master mix (BiotechRabbit, Milan, Italy). The amplification program was carried out on a 2720 Thermal Cycler (Applied Biosystem, Life Technologies, Milan, Italy) consisted of an initial denaturation of 5 min at 95 °C and 30 cycles of 30 s at 95 °C, 30 s at 65 °C, and 60 s at 72 °C with a final extension of 7 min at 72 °C. The resulting PCR amplicons were verified by comparison with the corresponding specific genes. *S. aureus* DSM 2569, *B. cereus* DSM 626, and *B. subtilis* DSM 10 were included in each PCR run as positive controls. Primer sequences are reported in Table S1.

2.4. LAB Strains and CFS Preparation

Four LAB strains, two *Lactiplantibacillus plantarum* (named AS1 and AS4) and two *Leuconostoc mesenteroides* (MEL1 and MAC1), were selected for their antibacterial performances against a panel of food spoilage bacteria [11]. In the present study, a mixture of the CFS from the four LAB strains was used. In detail, fresh culture of each LAB strain, grown in De Man—Rogosa—Sharpe (MRS: from Liofilchem srl) broth, at 32 °C, for 48 h, in anaerobic conditions, was centrifuged at 10,000× *g*, at 4 °C for 10 min, and then filtered with a 0.22 µm syringe cellulose acetate filter (LLG Labware, Meckenheim, Germany). A mixture of the single CFS from each strain was obtained and stored at 4 °C until use.

2.5. Antimicrobial Screening by Agar Diffusion Assay

The antimicrobial activity of the CFSs from the selected LAB strains was evaluated against *S. aureus* and *B. cereus*, following the procedures described by Ben Farhat and co-workers [21]. Inhibition was evidenced by a clear zone around the area of inoculation and measured from the center of the inoculation point to the edge of the inhibition, designated as the radius and measured in mm (≤5 mm, low inhibition; 5–10 mm, medium inhibition; ≥10 mm, high inhibition). Culture medium without cells served as a negative control. The agar diffusion assay was performed using the CFSs of each single LAB strain and a mixture of them, and screened against *S. aureus* DSM 2569, *B. cereus* DSM 626, and *B. subtilis* DSM 10, as positive controls, obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ; Braunschweig, Germany).

2.6. S. aureus and B. cereus Selection, and Inoculum Preparation

Among the isolates, two strains, one identified as *S. aureus* and one as *B. cereus,* were selected based on their highest sensitivity to the LAB antimicrobial effect and used in a challenge test on RTE orange slices. In detail, each strain was cultured in BHI broth (Liofilchem srl) and aerobically incubated at 37 °C overnight. A mixture of fresh cultures was then standardized to obtain a final density of 4 Log CFU/g of orange slices.

2.7. Processing and Treatments of Orange Slices

2.7.1. Vegetal Matrix

The orange fruits, belonging to the Navel cultivar, harvested in the winter of 2024, were kindly provided by the CREA experimental orchard, located in Palazzelli (Siracusa, Italy). The fruits were transferred to the Department of Agriculture, Food and Environment, at the University of Catania (Sicily) and immediately treated. The oranges were washed, dried, peeled, and cut into slices under aseptic conditions.

2.7.2. Inoculation, Packaging, and Storage Conditions

The challenge test was carried out following the procedures described by Foti and co-workers [11]. In detail, the orange slices (100 g) were placed under aseptic conditions in PS 6 transparent polystyrene bags (code: V00501/OPS) with the following characteristics: L: 127 × 115 mm; l: 45 mm; 0.08 m^3.

The inoculum of the target blend was prepared to reach a final density of 4 Log CFU/g of orange slices, with a 2:1 ratio of CFS to target bacteria. The CFS was obtained from a fresh culture of each LAB at 8 Log CFU/mL. Overall, eight experimental trials were performed, which included two single inoculated samples with the target reference strains (RB and RS), two single inoculated samples with the wild target strains (WB and WS), and two samples inoculated with the reference blend and the wild blend of target bacteria treated with the CFS (RBRS + CFS and WBWS + CFS). Additionally, one untreated sample (CTR) and one sample treated only with the CFS (CFS) obtained from the described LAB strains were used.

In Table 1, the sample nomenclature is reported. All samples were obtained in duplicate, stored at 4 °C for 14 days, and analyzed at the initial time (t0) and after 3 (t3), 8 (t8), and 14 (t14) days of storage. Physicochemical and microbiological analyses were performed at each sampling time.

Table 1. Sample nomenclature.

Code	RTE Orange Slices Treatment
CTR	Neither pathogen-inoculated samples, nor treated with CFS
CFS	CFS-treated samples
RB	Reference *B. cereus* strain-inoculated samples
RS	Reference *S. aureus* strain-inoculated samples
WB	Wild *B. cereus* strain-inoculated samples
WS	Wild *S. aureus* strain-inoculated samples
RBRS + CFS	Reference blend species-inoculated and CFS-treated samples
WBWS + CFS	Wild blend species-inoculated and CFS-treated samples

2.8. Analytical Parameters

2.8.1. Microbiological Analyses

To assess the levels of background microbiota and their dynamics during storage, total mesophilic count (TMC), *S. aureus,* total psychrophilic count (TPC), and *B. cereus* counts were determined. Specifically, 25 g of each sample (collected on the same day of inoculation, and after 3, 8, and 14 days of refrigerated storage) was homogenized using a lab blender for 120 s (Lab-Blender, Seward, London, UK) and serially ten-fold diluted. A volume of 0.1 mL from the appropriate dilutions was surface plated on PCA, MSA, and BHI agar media (Liofilchem srl, Italy), respectively. After incubation at specific conditions for each microbial group, the obtained colonies were analyzed for the conventional phenotypic features, as stated above.

2.8.2. Growth Potential (δ)

A predictive calculation of the possible outgrowth of inoculated microbial species was performed through the measurement of growth potential (δ). This refers to the logarithmic values of a bacterium's growth in a specific food over its shelf life, under programmed storage conditions (refrigeration and storage abuse) [22]. Specifically, the growth potential for the two bacterial species was determined by subtracting the initial inoculated concentration (Log_i) from the highest bacterial concentration recorded during the challenge test (indicated as Log_{max}). When δ is higher than 0.5 units, the matrix is classified as "able to support the growth of the selected strain"; if δ is ≤0.5 units, the matrix is classified as "unable to support the growth of the selected strain" [23].

2.8.3. DNA Extraction and qPCR Assay

Orange slice samples collected at 0, 3, 8, and 14 days of refrigerated storage were subjected to DNA extraction through a modified protocol using cetyl trimethyl ammonium bromide (CTAB) reagent (Fluka, BioChemiKa, Zola Predosa, Italy), according to Da Ma and coworkers [24], which included the bead beater treatment of samples for 3 × 60 s. DNA concentration was measured using the fluorimeter Qubit 4.0 (Invitrogen, Carlsbad, CA, USA). Then, DNA was subjected to qPCR following the method proposed by Oliwa-Stasiak and coworkers [25]. The qPCR reactions were performed using the WizPure qPCR Master (SYBR) kit (WizBioSolution, Seongnam, Republic of Korea) on a Rotor-Gene Q instrument (Qiagen, Milan, Italy). *S. aureus* quantification was carried out targeting the size variable *spa* gene region (ranging from 180–600 bp), and the *B. cereus* targeting the 186 bp *mot*B (Table S1).

In detail, two qPCR mixes were performed in a final volume of 20 µL, containing 10 µL of WizPure qPCR Master (SYBR), 10 µM of both forward and reverse primers, 2 µL of the DNA

template, and 6.8 µL of water. The cycling conditions were those previously reported. To ensure amplification specificity, a melting curve analysis was performed over a temperature range of 70 °C to 95 °C. To validate the reaction, a standard curve was generated using the genetic material isolated from the reference strains (*S. aureus* DSM 2569 and *B. cereus* DSM 626) in a range from 10^5 to 10^1 CFU/mL. All reactions were processed in three different replicates.

2.8.4. Physicochemical Analyses

At the beginning of the experiment and during refrigerated storage, different physical parameters were evaluated, as weight loss, color, and firmness.

The weight loss of orange slice samples was evaluated using a high-precision scale (Gibertini EU-C 2002 RS, Novate Milanese, Italy). In addition, the color of the samples was assessed at different storage intervals using a colorimetric method based on the standards established by the Commission Internationale de l'Éclairage (CIE). The parameters L* (brightness), a* (green-red), and b* (blue-yellow) were measured with a Minolta CM-2500d spectrophotometer (Minolta, Milan, Italy) according to the CIELAB system. For each repetition, the final color value was calculated as the average of three transmittance readings. Texture was evaluated with a texture analyzer (DO-FB0.5 TS 2002, ZwickRoell, Genoa, Italy) and expressed as the maximum force required to break the fruit skin. The instrument was equipped with a cylindrical probe (P8) with a diameter of 6.4 mm. The testing settings were as follows: pre-test speed 2 mm/s, test speed 0.5 mm/s, post-test speed 4 mm/s, a compression distance of 2 mm, and a maximum force threshold of 1 N. Three replicates, each consisting of five orange slices, were analyzed for each treatment. The peak force recorded during tissue rupture was used as an indicator of firmness and measured in Newtons (N).

The pH of the juice samples obtained from the orange slices was determined at each sampling point with a DL25 pH meter (Mettler-Toledo International Inc., Columbus, OH, USA). The content of total soluble solids (TSSs) was determined in samples using a refractometer (Atago RX-5000, Fisher Scientific, Rodano, Italy), and the values are reported in degrees Brix (°Brix).

2.9. Statistical Analyses

Statistical analysis was performed using a one-way analysis of variance (ANOVA), and Tukey's HSD post hoc test for means separation (significance level at $p \leq 0.05$), using the statistical software IBM SPSS Statistics for Windows, version 20 (IBM Corp., Armonk, NY, USA). All treatment values are presented as means ± SD. Moreover, the Bland–Altman plot was constructed by plotting the difference between the PC and qPCR measurement methods, as well as all graphs, using statistical software GraphPad PRISM, version 10.1.1 (270).

3. Results

3.1. Identification of Wild Target Bacteria from Commercial RTEF Samples

Based on phenotypic characterization, ten Gram- and catalase-positive rod-shaped, mannitol-fermenting, hemolytic, and lecithinase-producing isolates from RTE products (eight from oranges, one from pineapple, and one from cantaloupe) were considered to belong to the *Bacillus* group. At the same time, twelve Gram-positive, coagulase and catalase-positive, mannitol fermenting cocci isolates (six from oranges, two from cantaloupe, three from fruit salad, and one from pineapple) were grouped into the *Staphylococcus* group.

The PCR analyses, using the primers reported in Table S1 and the method reported in the previous section, confirmed the *B. cereus* affiliation for four isolates from oranges and for one isolate from pineapple, and the *S. aureus* affiliation for three isolates: two from oranges and one from cantaloupe.

3.2. Antimicrobial Activity of CFS

The antimicrobial activity of CFS from the four LAB strains previously described [11] against both indigenous and reference target bacteria of *S. aureus* and *B. cereus* was detected by agar diffusion assay. The tested CFSs showed appreciable antimicrobial activity against both species, confirming findings previously reported [11], with some differences between the two LAB species. Indeed, the CFSs obtained from *Lactiplantibacillus* sp. highlighted a significantly higher effect than those obtained from *Leuconostoc* sp., with a wider clear zone of inhibition. Moreover, no significant differences in antimicrobial effect were observed among strains belonging to the same genus.

3.3. Microbiological Analyses

Table 2 presents the microbial counts detected in the samples, and the Supplementary Figures S1 and S2 illustrate the survival dynamics of both *S. aureus* and *B. cereus* species on the RTE orange slices over time.

Table 2. Microbial counts in RTE orange slices.

Samples	Storage Days	Total Mesophilic Count (TMC)	*S. aureus*	Total Psychrophilic Count (TPC)	*B. cereus*
CTR	0	2.00 ± 0.01^{f}	2.00 ± 0.00^{f}	$<1^{e}$	2.30 ± 0.00^{c}
CFS	0	2.00 ± 0.00^{f}	2.00 ± 0.00^{f}	$<1^{e}$	$<1^{d}$
RB	0	2.00 ± 0.00^{f}	2.00 ± 0.00^{f}	2.00 ± 0.00^{d}	$<1^{d}$
RS	0	3.70 ± 0.01^{a}	3.52 ± 0.01^{a}	2.00 ± 0.01^{d}	$<1^{d}$
WB	0	3.48 ± 0.01^{c}	3.32 ± 0.00^{c}	2.30 ± 0.00^{c}	$<1^{d}$
WS	0	3.04 ± 0.02^{e}	3.24 ± 0.01^{d}	2.95 ± 0.00^{a}	3.50 ± 0.01^{a}
RBRS + CFS	0	3.54 ± 0.00^{b}	3.36 ± 0.00^{b}	2.00 ± 0.01^{d}	$<1^{d}$
WBWS + CFS	0	3.28 ± 0.00^{d}	2.95 ± 0.00^{e}	2.90 ± 0.00^{b}	2.30 ± 0.00^{c}
CTR	3	2.00 ± 0.00^{g}	2.30 ± 0.01^{e}	2.85 ± 0.00^{e}	3.00 ± 0.01^{b}
CFS	3	4.36 ± 0.01^{b}	2.00 ± 0.00^{f}	3.30 ± 0.00^{b}	2.30 ± 0.01^{d}
RB	3	2.48 ± 0.00^{f}	$<1^{g}$	2.90 ± 0.01^{d}	$<1^{f}$
RS	3	3.41 ± 0.00^{d}	$<1^{g}$	2.30 ± 0.00^{h}	2.48 ± 0.02^{e}
WB	3	3.23 ± 0.03^{e}	3.26 ± 0.01^{b}	2.78 ± 0.00^{f}	$<1^{f}$
WS	3	5.19 ± 0.00^{a}	3.19 ± 0.00^{c}	3.20 ± 0.02^{c}	$<1^{f}$
RBRS + CFS	3	3.72 ± 0.01^{c}	3.11 ± 0.02^{d}	3.85 ± 0.00^{a}	3.48 ± 0.00^{c}
WBWS + CFS	3	4.40 ± 0.00^{b}	6.30 ± 0.00^{a}	2.70 ± 0.00^{g}	3.90 ± 0.02^{b}
CTR	8	3.54 ± 0.00^{g}	5.48 ± 0.00^{c}	5.70 ± 0.00^{d}	3.54 ± 0.02^{a}
CFS	8	5.68 ± 0.00^{d}	7.07 ± 0.01^{a}	4.96 ± 0.00^{f}	$<1^{d}$
RB	8	2.00 ± 0.00^{h}	2.30 ± 0.00^{e}	4.64 ± 0.00^{h}	$<1^{d}$
RS	8	6.45 ± 0.00^{b}	$<1^{g}$	6.32 ± 0.00^{b}	$<1^{d}$
WB	8	4.29 ± 0.01^{f}	4.30 ± 0.00^{d}	4.80 ± 0.00^{g}	$<1^{d}$
WS	8	7.89 ± 0.00^{a}	2.30 ± 0.00^{e}	7.43 ± 0.00^{a}	$<1^{d}$
RBRS + CFS	8	5.93 ± 0.00^{c}	2.00 ± 0.00^{f}	5.40 ± 0.00^{e}	2.00 ± 0.00^{c}
WBWS + CFS	8	4.36 ± 0.00^{e}	7.02 ± 0.02^{b}	5.78 ± 0.00^{c}	2.48 ± 0.01^{b}
CTR	14	4.37 ± 0.01^{f}	3.08 ± 0.00^{b}	5.86 ± 0.00^{h}	4.66 ± 0.00^{a}
CFS	14	7.65 ± 0.00^{c}	$<1^{e}$	7.66 ± 0.00^{c}	$<1^{d}$
RB	14	3.20 ± 0.02^{g}	2.00 ± 0.00^{d}	6.51 ± 0.02^{f}	3.20 ± 0.01^{b}
RS	14	8.31 ± 0.01^{b}	$<1^{e}$	7.00 ± 0.01^{e}	$<1^{d}$
WB	14	5.09 ± 0.00^{e}	2.70 ± 0.00^{c}	6.48 ± 0.00^{g}	4.78 ± 0.05^{a}
WS	14	7.66 ± 0.00^{c}	$<1^{e}$	7.94 ± 0.00^{b}	$<1^{d}$
RBRS + CFS	14	8.39 ± 0.01^{a}	$<1^{e}$	8.11 ± 0.00^{a}	$<1^{d}$
WBWS + CFS	14	6.73 ± 0.01^{d}	5.35 ± 0.01^{a}	7.15 ± 0.00^{d}	2.48 ± 0.01^{c}

Data are presented as mean Log CFU/g ± standard deviation, based on 2 replicates. Different superscript letters within the same column indicate significant differences at $p < 0.05$ between the samples for each sampling time.

Overall, the results exhibited high variability among the samples ($p \leq 0.05$). Specifically, at the initial time (t0), the TMC ranged from 2.0 to 3.7 Log CFU/g, with the lowest values detected in CTR, CFS, and RB samples. After three days, all samples maintained the same cell densities, except the CFS and the WS, where the mean TMC values increased to 4.36 and 5.19 Log CFU/g, respectively (Table 1). As expected, starting from the 8th day of storage,

the TMC density dramatically increased, particularly in WS and in RBRS + CFS samples, reaching the highest values after 8 and 14 days, respectively. Fluctuations in the *S. aureus* count were observed. While the initial counts aligned with those of the TMC, the *S. aureus* cellular density increased in the first days and then decreased, dipping below detection limits (Table 2). After 14 days, the mean count was nearly equal to the initial value, except for the WBWS + CFS sample, where a 5.3 Log CFU/g value was detected. Results for TPC indicated a significant difference between CFS-treated (inoculated with reference and indigenous strains) and untreated samples, with the CTR and CFS samples showing initial values below detection limits and the other samples a mean count of 2.00 Log CFU/g. Starting from the 8th day of refrigerated storage, the mean count increased till the end of storage, sometimes exceeding 7.00 Log CFU/g (Table 2). The initial count of *B. cereus* was higher in WS and WBWS + CFS samples, reaching 3.50 and 2.30 Log CFU/g, respectively, while the highest value (4.78 Log CFU/g) was detected in the WB sample after 14 days. For the other samples, the presence of *B. cereus* was sporadically noted with a mean value of 3.00 Log CFU/g (Table 2).

The different behaviors of reference and indigenous strains belonging to the two target bacterial species in the tested matrix treated with the CFS, is compared in Figure 1, where the difference, at 0, 3, 8 and 14 days, between the Log CFU/g detected in samples RB or WB and Log CFU/g detected in CTR sample are reported (Figure 1, panel a and b, respectively). As shown, an overall higher and faster inhibitory effect against both species was detected in the samples inoculated with the reference blend (RBRS + CFS). Indeed, for the RBRS + CFS and WBWS + CFS samples, only *S. aureus* represented a part of the indigenous microbiota, demonstrating an initial increase in counts after 3 days of storage in the RBRS + CFS sample, becoming more pronounced in the WBWS + CFS, where it persisted until the end of storage (Figure 1, panel b). *B. cereus*, although initially present in the RBRS + CFS sample and well-adapted in WBWS + CFS, after 3 days of storage, underwent a sharp reduction in the remaining sampling points in both samples.

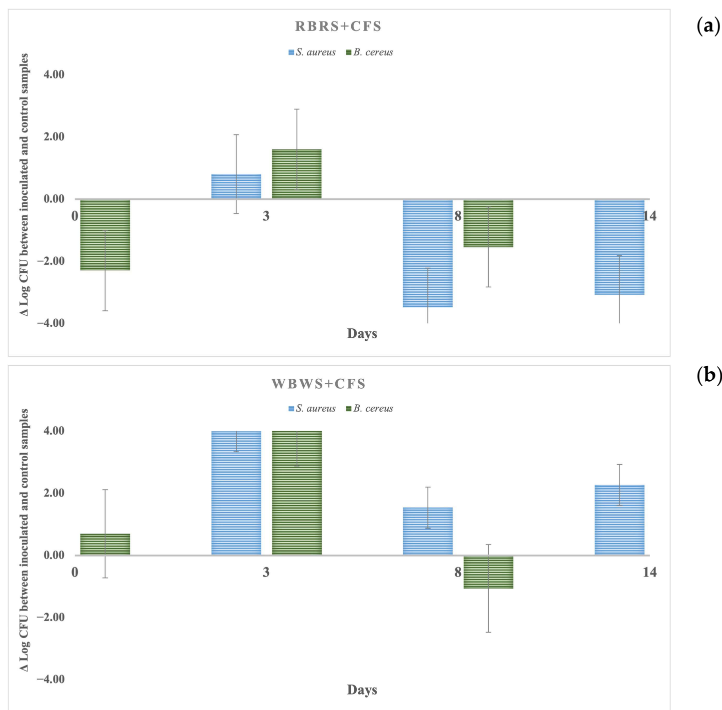

Figure 1. Ability to proliferate of the reference (panel **a**), and the wild (panel **b**) target bacterial species in the tested matrix treated with CFS.

3.4. Growth Potential (δ)

The growth trend of both reference and wild *S. aureus* and *B. cereus* strains (as Log CFU/g) in inoculated RTE orange slices samples during storage at 4 °C, from the initial time to the 14th day, is shown in Tables 3 and 4, respectively. As shown in Table 3, differences in δ were observed among the four samples inoculated with *S. aureus*. The sample with the highest growth potential was the sample inoculated with the blend of reference *S. aureus* (RSA sample), not treated with CFS.

Table 3. Growth potential of both reference and indigenous *S. aureus* inoculated in RTE orange slices calculated considering the initial inoculum (as 4 Log CFU/g).

Samples	***S. aureus* Density (Log CFU/g)**				**Growth Potential δ**
	Day 0	**Day 3**	**Day 8**	**Day 14**	
WBWS + CFS	2.95	6.30	7.02	5.35	3.07
RBRS + CFS	3.36	3.11	2.00	0.00	−0.64
WS	3.24	3.19	2.30	0.00	−0.76
RS	3.70	3.41	6.45	8.31	4.31

Table 4. Growth potential of both reference and wild *B. cereus* inoculated in RTE orange slices, calculated considering the initial inoculum (as 4 Log CFU/g).

Samples	***B. cereus* Density (Log CFU/g)**				**Growth Potential δ (Log CFU/g)**
	Day 0	**Day 3**	**Day 8**	**Day 14**	
WBWS + CFS	0.00	3.48	3.90	6.60	2.60
RBRS + CFS	2.30	0.00	2.48	0.00	−1.52
WB	4.66	0.00	3.20	0.00	0.66
RB	2.00	2.48	2.00	3.20	−0.80

Regarding *B. cereus* (Table 4), only the sample inoculated with the wild blend and CFS treated (WBWS + CFS) effectively supported its growth, showing the highest δ. The WB sample provided slight support for *B. cereus* proliferation.

3.5. q-PCR Assay

In Figure 2 (panels a and b), the C_q values related to cell concentration (Log DNA copies/g) of *S. aureus* and *B. cereus* in RTE oranges after 0, 3, 8, and 14 days of refrigerated storage, are reported. The standard curves for both species indicated a linear relationship, in accordance with the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines [26]. To ensure the reliability of the analyses, the standard curves were evaluated based on an R2 value greater than 0.95 and a slope between −3.0 and −3.9, corresponding to PCR efficiencies between 80% and 115%. The detection limits of the two target bacterial species were determined using 10-fold serial dilutions of the DNA extracted from the bacterial suspension. For *S. aureus*, the established linear relationship was CT = −2.570 + 18.853, with an R^2 of 0.99765 and an efficiency of 99%, resulting in 5 orders of magnitude improvement. For *B. cereus*, the established linear relationship was y = −2.692 + 12.499, with an R^2 of 0.99909 and an efficiency of 91%, leading to a reduction of 5 orders of magnitude.

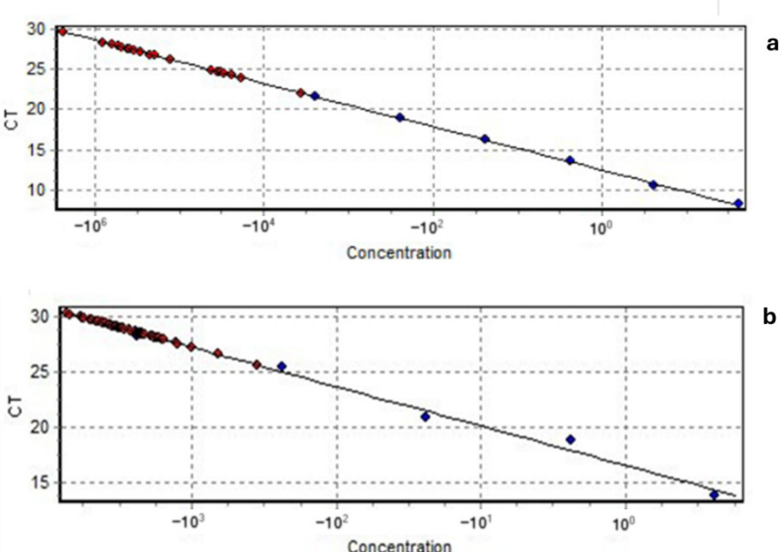

Figure 2. Standard curve for quantification of bacteria in RTE orange. *Ct* values by real-time PCR were plotted against the genetic material isolated from the reference strains: (**a**) *S. aureus* DSM 2569 and (**b**) *B. cereus* DSM 626.

The presence of *S. aureus* and *B. cereus* was confirmed in 28 out of 32 samples (87.5%) and 27 out of 32 samples (84%), respectively. *S. aureus* quantification by qPCR ranged from 3.48 to 3.90 Log DNA copies/g. Only four samples (CTR and CFS from days 0 and 3) fell outside the calibration curve range, with values below 3.5 Log DNA copies/g. *B. cereus* qPCR quantification ranged from 1.65 to 4.84 Log DNA copies per gram. Likewise, the previously mentioned samples were outside the calibration curve range, with values below 1.65 Log DNA copies/g.

The microbial density across the two intentionally inoculated pathogens detected through qPCR (blue lines) and plate count (red lines) methods is shown in Figure 3.

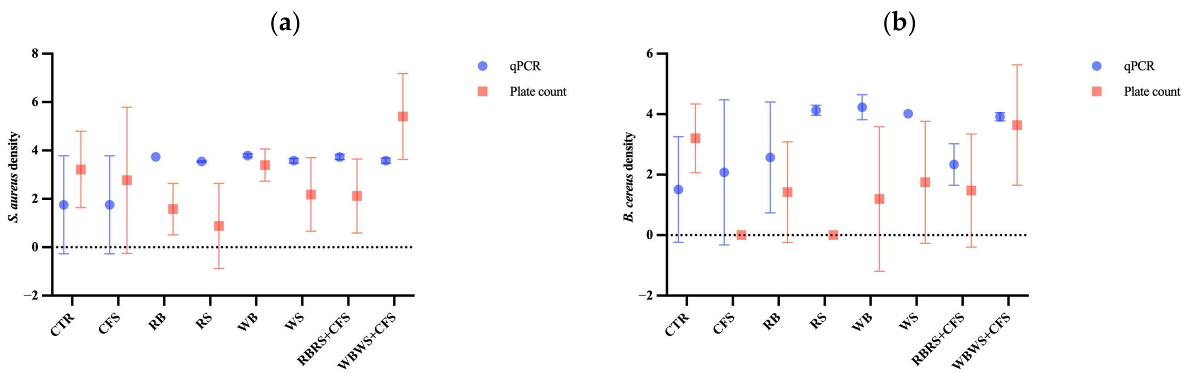

Figure 3. Comparison of microbial densities of *S. aureus* (**a**), and *B. cereus* (**b**) detected by qPCR and plate counting.

Regarding *S. aureus*, the microbial load detected by qPCR was generally higher than that determined by plate counting, except for the CTR, the CFS, and the WBWS + CFS samples, where it was lower. Furthermore, for *S. aureus*, the variability in qPCR data was lower compared to that observed in plate counting. Conversely, for *B. cereus*, higher variability was observed. Indeed, for both techniques, significant differences throughout the experimental period were observed ($p \leq 0.05$), with an overall microbial load slightly lower but more variable in both cases (Figure 3, panel b). To better understand the reliability and agreement between the two detection techniques, a heatmap is reported in Figure 4.

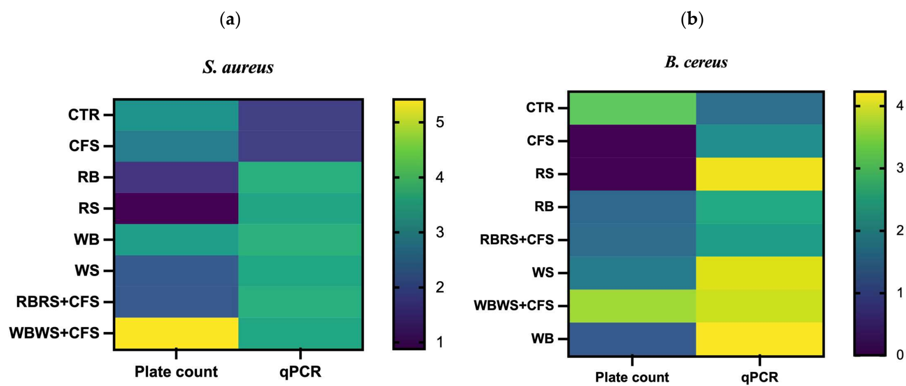

Figure 4. Heatmaps comparing the two detection methods during the experimental period for each sample, for *S. aureus* (**a**), and *B. cereus* (**b**). Results are expressed as the means of sampling times, in each sample, where the target bacterial cellular densities span from 0 to 6 Log CFU/g.

Overall, the heatmap suggested that there is no uniform correlation between the plate counting (PC) and qPCR methods across all samples, while a more uniform pattern compared to the PC column was registered for both species in the qPCR method. For *S. aureus*, the qPCR methods revealed higher values with higher sensitivity in all samples, except for the WBWS + CFS samples, where lower values were detected compared to plate counting.

Finally, predominantly features darker shades of blue and teal in *B. cereus* detection, suggesting weaker correlations between PC and qPCR methods across most samples.

Lastly, Bland–Altman plots (Figure 5) were used to evaluate the agreement between the methods. The upper and lower horizontal lines represent the 95% limits of the agreement. The limits ranged from −2.91 to 3.89 Log CFU/g and −2.00 to 5.03 Log CFU/g (Figure 5b) for *S. aureus* and *B. cereus* (Figure 5, panel a and panel b), respectively.

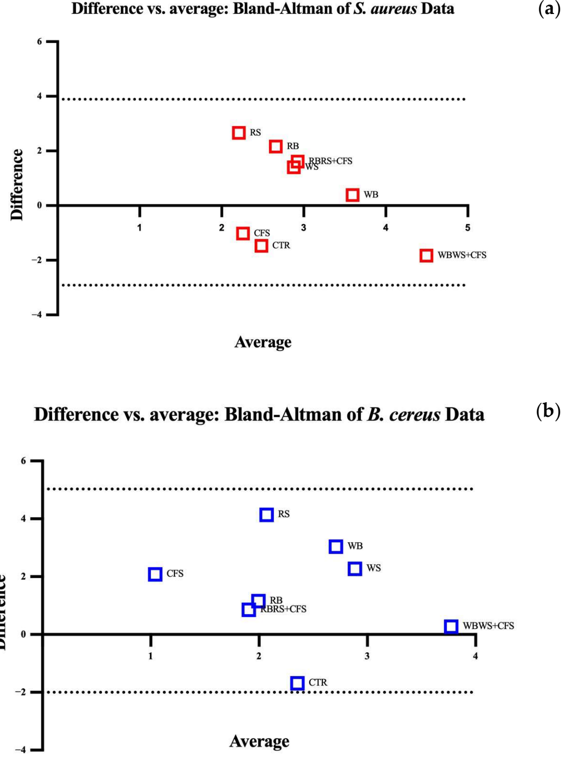

Figure 5. Bland–Altman analysis of agreement of cell concentration values obtained by PC and qPCR methods in RTE orange slices for *S. aureus* (**a**), and *B. cereus* (**b**).

These results indicate that 95% of the pairs of enumeration results for *S. aureus* and *B. cereus* differed by less than 2.91 and 2.00 Log CFU/g, respectively. This suggests that the PC method generally yielded lower values compared to the qPCR method [Log CFU/g (PC − qPCR)]. For both species, in the plots, the data points are scattered around the mean difference lines, with most falling within the limits of agreement. This suggests a general agreement between the two methods, although some variability is evident.

3.6. Physicochemical Analyses

At the initial time, the fresh orange slice samples exhibited a pH value of 3.68, and all samples showed an increase in pH over time (Figure 6, panel a). In particular, the RBRS + CFS samples showed the highest pH value (4.54) after 14 days. In contrast, the WBWS + CFS sample showed an initial decrease in pH value, which reached the lowest value of 3.90 after 8 days, followed by a further increase up to 14 days. Overall, the TSS values (Figure 6, panel b) remained relatively stable over time in all samples, showing for the same samples a slight decrease between the 3rd and the 14th day.

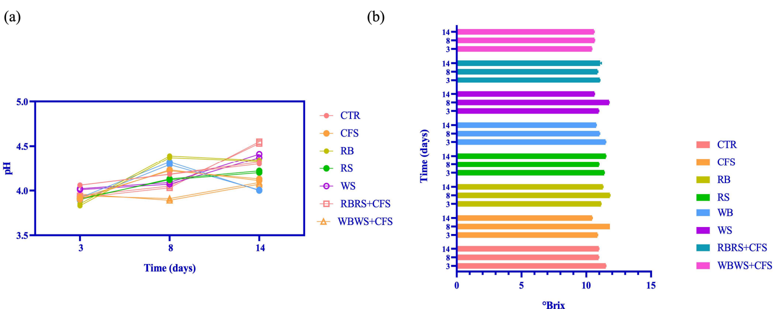

Figure 6. Chemical parameters detected in RTE orange slice samples: (**a**) pH and (**b**) total soluble solids (TSSs) expressed as °Brix.

The results related to the weight loss revealed that, during refrigerated storage, in all samples, an increase in weight, without any statistical difference, was observed, which could be related to the development of condensation and moisture retention.

During the storage, color and texture parameters were monitored in all samples (Table 5). Concerning color, all colorimetric coordinates showed the greatest statistically significant difference after 3 days. In particular, the L* (brightness) showed significant differences among the treatments only at the 3rd day, when the WBWS + CFS sample recorded the value of 51.03, conferring a brighter appearance to the orange slices than the RB, which showed the lowest values. However, on days 8 and 14, these differences tended to decrease, suggesting that the initial difference among the treatments progressively disappeared.

Table 5. Color and texture coordinates of RTE orange slices during storage time.

Samples	Storage Days	L* (D65)	a* (D65)	b* (D65)	Firmness (N)
Fresh orange slices	0	48.98 ± 2.78	-3.10 ± 0.47	32.73 ± 3.69	4.94 ± 1.00
CTR	3	47.82 ± 1.84^{ab}	-2.54 ± 0.75^{a}	34.38 ± 2.77^{ab}	3.51 ± 0.83
CFS	3	46.70 ± 1.51^{ab}	-2.61 ± 0.48^{a}	32.31 ± 1.36^{ab}	3.89 ± 0.80
RB	3	45.92 ± 2.30^{b}	-2.64 ± 0.75^{a}	26.30 ± 1.68^{c}	3.81 ± 1.18
RS	3	47.60 ± 0.53^{ab}	-4.68 ± 0.43^{b}	29.36 ± 1.59^{bc}	4.21 ± 1.01
WB	3	47.54 ± 2.31^{ab}	-3.04 ± 1.20^{a}	31.98 ± 2.29^{ab}	4.12 ± 1.30
WS	3	48.91 ± 2.30^{ab}	-2.38 ± 1.21^{a}	33.90 ± 2.28^{ab}	3.92 ± 0.66
RBRS + CFS	3	46.24 ± 3.81^{ab}	-2.24 ± 1.11^{a}	30.19 ± 2.56^{bc}	4.59 ± 0.88
WBWS + CFS	3	51.03 ± 0.95^{a}	-3.47 ± 0.31^{ab}	35.70 ± 2.32^{a}	3.33 ± 0.81

Table 5. *Cont.*

Samples	Storage Days	L* (D65)	a* (D65)	b* (D65)	Firmness (N)
		*	*	**	n.s.
CTR	8	48.21 ± 2.07	−3.65 ± 0.91	28.93 ± 2.80 ab	4.89 ± 1.30 a
CFS	8	46.43 ± 2.94	−2.47 ± 1.09	28.03 ± 4.36 ab	4.27 ± 0.89 ab
RB	8	45.75 ± 1.89	−2.69 ± 4.23	25.86 ± 2.73 ab	4.43 ± 1.08 ab
RS	8	44.47 ± 2.02	−3.46 ± 0.87	25.90 ± 3.37 ab	3.35 ± 1.00 b
WB	8	47.43 ± 2.18	−3.98 ± 1.23	31.65 ± 2.84 a	4.36 ± 1.29 ab
WS	8	45.25 ± 0.79	−3.32 ± 0.35	23.97 ± 2.68 b	4.19 ± 1.10 ab
RBRS + CFS	8	46.06 ± 1.15	−3.47 ± 0.86	30.17 ± 2.49 a	4.10 ± 0.73 ab
WBWS + CFS	8	46.76 ± 1.97	−3.79 ± 1.02	28.27 ± 1.88 ab	4.64 ± 1.16 ab
		n.s.	n.s.	*	*
CTR	14	45.10 ± 0.97	−3.15 ± 0.64 ab	30.19 ± 1.84	5.57 ± 1.38
CFS	14	47.97 ± 2.09	−2.68 ± 0.50 ab	29.01 ± 2.16	4.89 ± 0.86
RB	14	50.01 ± 2.11	−4.92 ± 0.78 c	32.47 ± 1.00	5.00 ± 1.04
RS	14	49.91 ± 1.98	−2.25 ± 0.57 a	33.03 ± 0.84	4.90 ± 0.94
WB	14	49.83 ± 3.88	−3.77 ± 0.45 bc	30.74 ± 4.42	4.89 ± 1.34
WS	14	47.90 ± 1.91	−3.13 ± 0.98 ab	30.37 ± 3.37	4.78 ± 1.36
RBRS + CFS	14	48.98 ± 4.25	−3.49 ± 0.78 ab	34.12 ± 4.81	4.40 ± 1.47
WBWS + CFS	14	48.99 ± 2.44	−3.37 ± 0.62 ab	29.13 ± 2.45	4.74 ± 1.16
		n.s.	**	n.s.	n.s.

Data are expressed as means ± SD. Different letters in the same column for each sampling time indicate significant differences between the means (n = 3) based on Tukey's test ($p \leq 0.05$). L* (brightness), a* (green–red component), and b* (blue–yellow component). n.s. not significant. * Significance at $p \leq 0.05$. ** Significance at $p \leq 0.01$.

As for the a* component, indicative of green–red hue, the RS and RB samples showed a marked green hue: in particular, RS and RB, after 3 and 14 days, respectively, showed significantly more negative values than the other treatments. The b* component, representing the yellow color, also showed significant changes, especially after 3 and 8 days. The WBWS + CFS sample maintained a more intense yellow hue on the first days, suggesting a better preservation of the typical orange appearance. In contrast, the RB samples showed lower values, at 26.30 after 3 days, indicating a loss of the yellow component. The texture showed no significant differences after 3 and 14 days. However, on the 8th day, some differences were noticeable. Indeed, the CTR sample was the most consistent, while the RS showed a significantly lower consistency. This could indicate that some treatments, such as RS, tend to slightly compromise the firmness of the fruit in the intermediate refrigerated storage period.

4. Discussion

Despite the health benefits of fruit and vegetable consumption, safety remains a concern for RTEF products, as they have been identified as potential carriers of foodborne pathogens [27]. Indeed, along with a variety of enteric pathogens, such as *E. coli*, *Salmonella* spp., and *L. monocytogenes* involved in RTE-associated diseases, *S. aureus* and *B. cereus* have been frequently reported in previous studies, representing relevant food safety hazards [5,14,28]. Therefore, ensuring microbiological quality and compliance with standards set by Regulation (EC) No 2073/2005 is crucial for food producers, especially for species as *B. cereus*, able to cause illness at concentrations less than or equal to 10^3 CFU/g, and therefore unlikely to be detected with cultivation-based methods, and for which legislation does not require routine screening [14,15]. Moreover, given the spore-forming nature of *B. cereus*, its survival during refrigerated storage is plausible and poses a potential risk in RTE products. Indeed, despite the refrigerated storage of many RTE fruit and vegetable products, under inadequate storage conditions (e.g., increased temperature, prolonged storage, slicing and

packaging that increase nutrient availability), spores may germinate, leading to bacterial growth and potential toxin production, resulting in foodborne illnesses [9,15].

Although culture-based methods remain the reference to ensure compliance with established regulatory food safety thresholds, effective detection is still a challenging task, due to interference from other non-target microbiota, low numbers of target groups, and the interaction between microorganisms and food matrices [29]. Quantitative PCR (qPCR) is a relevant technique to detect and quantify key pathogens in food; it also enables the detection of non-cultivable bacteria and subdominant populations or very low-level cells [15,28].

In this context, the microbiological challenge combined with a molecular-based approach offers a valuable tool to assess safety traits of RTE products [30]. In the present study, the suitability of the CFS from wild LAB with proven antimicrobial capabilities was evaluated against wild isolates of *S. aureus* and *B. cereus*, during refrigerated storage, using plate count and qPCR analyses.

Although RTE orange slices, due to their intrinsic features mainly related to pH, harbor modest native microbiota, our microbiological data indicated the presence of a background microbiota, as well as their ability to support the growth of the two bacterial species intentionally inoculated. Overall, microbiological results revealed significant differences among microbial counts, with an initial microbial population consisting of total mesophilic bacteria and total psychrophilic bacteria, *S. aureus*, and *B. cereus*, occurring below legislative limits. After 3 days, as expected, the microbial loads increased, revealing high, unsatisfactory bacterial loads in all samples. In control samples, *S. aureus* and *B. cereus* were found at low densities over the experimental time. Generally, the growth of wild *S. aureus* and *B. cereus* was supported in all samples, highlighting the better growth potential of a native microbial population. In the case of the RBRS + CFS sample, their load remained constant and always below the inoculation value, disappearing completely by the 14th day, which highlights the greater effectiveness of CFS on reference strains compared to indigenous strains and confirms the ANSES EURL Lm ISO 20976-1 purpose guidelines requiring the use of wild strains [16]. Several studies [10,11,21,31] investigating the microbiological safety of RTE fruits assessed the suitability of CFS as a biopreservative. Indeed, some organic acids have been described as antimicrobials or antioxidants by slowing or preventing changes in color, flavor, texture, and delaying rancidity [12].

The combination of conventional plate counting and qPCR analyses enabled us to obtain a comprehensive assessment of the microbial dynamics in RTE orange slices. Here, the qPCR approach proved suitable for detecting and quantifying both *S. aureus* and *B. cereus* in RTE orange slices. Moreover, the Bland–Altman analysis provided a visual and statistical means to assess the agreement between the two methods, demonstrating a tendency for agreement between the two methods for both *S. aureus* and *B. cereus*, even though some outliers specifically related to sample conditions or inherent differences in the measurement techniques were identified. Our results aligned with those of other studies, utilizing molecular methods as a faster and combined tool to quantify both the overall microbial and pathogenic load in RTE food [32–35]. Ultimately, the analyses demonstrated that plate counting consistently measured lower values than qPCR, especially for B. *cereus*, highlighting the need for an additional analytical method to detect bacteria at very low concentrations. A possible reason for these results is that the plating counting does not account for the enumeration of cells in the viable but non-culturable (VBNC) state and under stress. Indeed, microorganisms in food are often affected by a variety of processing hurdles, which may lead to the loss of their ability to grow on selective media—cultivability—while viability remains unaltered, becoming VBNC. In general, adverse conditions, including nutrient starvation, low temperature, acidic treatment, oxygen stress, and others, are

relevant for the induction of VBNC, as already demonstrated for both *S. aureus* and *B. cereus* species [36,37]. For example, nutrient starvation treatment of *S. aureus* revealed that colonies enter the VBCN state within a short time, becoming VBNC even faster with a hostile pH environment or salt concentration. Likewise, despite many advantages, qPCR still faces some drawbacks, such as food matrix inhibitors and the inability of the methods to discriminate between viable and dead cells, which often results in an overestimation of the target microorganisms as well as the generation of nonspecific amplification products [35]. Several studies have introduced methods to distinguish between viable and dead bacterial cells, such as the use of intercalating dyes (propidium monoazide and ethidium monoazide); implementation of viability PCR; and pre-rRNA analysis utilized to concentrate the target organism from a relatively large sample size, remove potential PCR inhibitors, and yield samples in a volume suitable for qPCR [33].

During refrigerated storage, significant changes occurred in the chemical and physical parameters of the orange slices, with variations in pH, color, and texture closely related to the treatments applied. The progressive increase in pH in the different treatments suggests that microbial metabolic activity could affect organoleptic quality over time. Overall, the data showed that the different treatments selectively influence the physicochemical structural stability of orange slices, with important implications for the shelf-life and final quality of the product [11]. The WB + CFS samples were characterized by a better preservation of colorimetric properties, in particular brightness (L*) and yellow intensity (b*), especially in the early stages of storage, suggesting a more stable appearance. Instead, as reported by Zhang and coworkers [38], in RSA samples, a significant reduction in texture halfway through storage, probably linked to enzymatic or microbial metabolic processes that damaged the fruit's tissue structure.

5. Conclusions and Future Perspectives

Foodborne pathogens are often present in fresh-cut fruits and vegetables, contributing to foodborne outbreaks. This study examined the growth potential of wild *B. cereus* and *S. aureus* isolates, which were deliberately inoculated onto RTE orange slices, to evaluate the effectiveness of wild LAB CFS as a biopreservative. Overall, the significance of this research lies in improving the food safety of RTE orange slices, a product never before studied in this respect. Despite the natural attributes of oranges, our results indicated the presence of background microorganisms, and that orange slices can support the growth of the target bacteria intentionally inoculated. The results demonstrate that wild bacteria, compared to the reference ones, can grow more effectively. The study also emphasized the importance of combining plate counting with qPCR approaches to detect *B. cereus*, which can be risky even at low levels.

Finally, further research is needed to explore novel LAB candidates as bioprotective agents against a broader group of microorganisms and to understand the mechanisms of action of CFS. Furthermore, to optimize any bioprotective application for improving safety and prolonging the shelf-life of RTE fruits in the horticultural agri-food industry, economic feasibility studies at the industrial level need to be conducted, and regulatory approval granted.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation11110617/s1, Table S1: Primer sequences used in the present study. Figure S1: Survival dynamics of *S. aureus* species on RTE orange slices over time; Figure S2: Survival dynamics of *B. cereus* species on the RTE orange slices over time.

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