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Lactic Acid Bacteria and *Bacillus subtilis* as Potential Protective Cultures for Biopreservation in the Food Industry

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Abstract: The use of bacteria and/or their compounds is an alternative to the use of positive-list additives that the food industry is using as a tool to meet consumer demands for more natural, long-shelf-life, and healthy products, in short, to offer clean label foods. The aim of this study is to investigate the suitability of cell-free supernatants (CFSs) from Qualified Presumption of Safety strains as bioprotective cultures. Out of an initial screening panel of about 200 isolates, strains *Pediococcus acidilactici* CNTA 1059, *Lactiplantibacillus plantarum* CNTA 600, *Levilactobacillus brevis* CNTA 1374, and *Bacillus subtilis* CNTA 517 demonstrated strong antimicrobial activity against, especially, Gram-positive bacteria. The CFSs of these four strains showed minimum inhibitory concentration values between 0.15% and 5% against *Listeria monocytogenes* and *Lentilactobacillus parabuchneri*. None of the four selected strains exhibited acquired resistance to target antibiotics, and the non-toxigenicity of all the CFSs was demonstrated. In the case of the three lactic acid bacteria, the presence of bacteriocin-like inhibitory substances was confirmed following the decline in antimicrobial activity due to treatment with proteases. Regarding *B. subtilis*, biosynthetic gene clusters for different bacteriocin-like substances, including protease-resistant lipoproteins, were found via whole-genome sequencing. In addition, all of the CFSs exhibited stable antimicrobial activity at a wide range of temperatures (70–121 °C) used for the pasteurization and sterilization of food products, with a loss of antimicrobial activity ranging from 3% to 28%. These results point to the possibility that CFSs from these strains could be used in the food industry as a biocontrol tool to develop new products.

Keywords: biopreservation; protective cultures; lactic acid bacteria; *Bacillus subtilis*; thermorresistance

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1. Introduction

Consumer trends evolve along with technology and science. In this context, more and more consumers are becoming aware of the importance of a healthy and sustainable diet, which transpires to their dietary choices. The rise of "clean label" products epitomizes this trend, emphasizing attributes like additive-free formulae, short ingredient lists, transparent information, and health benefits [1–3]. However, all food has to be safe for consumption, necessitating measures to mitigate the presence of undesirable pathogenic micro-organisms such as *Escherichia coli*, *Listeria monocytogenes*, and *Salmonella* spp. [4]. Additionally, food waste is an additional challenge, particularly due to spoilage micro-organisms like *Penicillium* spp. and *Aspergillus* spp. [5]. Considering this, preservation is a fundamental step in food production processes worldwide, required to guarantee food safety and extend shelf life. On the one hand, conventional preservation technologies employed by the food industry encompass various methods, including heat treatments (such as pasteurization and sterilization), cooling techniques (like refrigeration and freezing), the reduction in water activity (such as spray-drying or freeze-drying), fermentation, and the use of chemical preservatives [6]. Nonetheless, most of these traditional approaches

commonly modify both the nutritional and organoleptic properties of food products, so they are not fully aligned with current consumer preferences and trends [7].

A variety of novel preservation strategies have arisen in response to the clean-label production trend, such as the use of bioprotective bacterial cultures and their metabolites [8–10]. This entails the use of micro-organisms and their products (such as their cell-free supernatants, CFSs) to increase the shelf life of food through competitive exclusion or the production of antimicrobial substances [9,10]. Only microbial species included in the Qualified Presumption of Safety list (QPS) [11] can be used to produce bioprotective cultures, and bacteria should not harbor any acquired transferrable antimicrobial resistance genes to clinically relevant antimicrobials to avoid horizontal transfer to other bacteria [12]. Additionally, and in line with EFSA's guidance on the characterization of production micro-organisms used in feed [13], there should be evidence of the non-toxigenicity of the cultures and of the stability of the intended technological effect, along with the definition of the quantity to be used at safe levels [10,14,15].

Among possible protective micro-organisms, lactic acid bacteria (LAB) stand out as a compelling choice: they boast a well-established tradition in food applications, are perceived as safe for human consumption, and exhibit a broad spectrum of antimicrobial activity [10]. The antimicrobial capacity of LAB is attributed to the synthesis of various antimicrobial compounds, such as organic acids, hydrogen peroxide, and bacteriocins [16]. Organic acids result from the fermentation of carbohydrates within the surrounding medium or matrix. This generates an adverse environment, preventing microbial proliferation and growth [17]. In aerobic conditions devoid of intracellular catalase, pseudocatalase, and peroxidase, LAB can produce hydrogen peroxide (H_2O_2). This compound leads to the degradation of cellular components, including membrane proteins (SH group oxidation) [16], and the infliction of DNA damage upon the targeted micro-organism [18]. Lastly, bacteriocins, which are ribosomal peptides, exhibit antimicrobial activity whose spectrum varies depending on the specific type of bacteriocin. Additionally, they have demonstrated notable thermoresistance and resilience to pH modifications [19].

In addition to LAB, interest in the genus *Bacillus* has grown due to its multiple biological activities, including antifungal and antimicrobial activity [20]. They are considered QPS strains [11] able to produce different compounds, including bacteriocins. These have been classified depending on their size and post-translational modification in a different classification to the bacteriocins of LAB [21]. The mechanisms of action of these bacteriocins are diverse, among which include the induction of membrane permeabilization in target micro-organisms through the release of enzymes and antimicrobial compounds, inducing alterations in the plasma membrane responsible for delineating the intracellular compartment from the extracellular environment; impeding vital cellular survival processes upon plasma membrane traversal; and intervening in the quorum sensing process associated with pathogen virulence attributed to the motility, biofilm formation, and cell reproduction [22]. Nevertheless, a substantial need for comprehensive information regarding the safety and spectrum of activity is required for its utilization [23] because their activity varies depending on the structure [20].

The main objectives of this work were (i) to select QPS-compliant species from our microbial collection that can produce cell-free supernatants (CFSs) with antimicrobial activity, (ii) to study whether the selected strains are free of acquired antibiotic resistance and are not cytotoxic, (iii) to elucidate the nature of their antimicrobial activity, and (iv) to assess their suitability for application in foods that are subjected to pasteurization or sterilization processes.

2. Materials and Methods

2.1. Selection of Potential Bacterial Strains as Protective Cultures

The CNTA (Centro Nacional de Tecnología y Seguridad Alimentaria) collection encompasses 200 bacterial strains of 24 bacterial species as potential protective cultures, including *Bacillus* sp. (1 species), *Bifidobacterium* sp. (4 species), and lactic acid bacteria

(LAB) (19 species) (Table S1). All of them belong to the QPS list and their identity has been individually verified via 16S rRNA gene sequencing. Table S1 also includes the origins of isolation of each genus.

The assays of the antimicrobial activity of the protective cultures were carried out using the CFSs of the cultures, which were obtained under optimal growth conditions for each micro-organism (Table S2).

To obtain the CFSs, the cultures were centrifuged at 4347× *g* for 10 min at 4 °C and the supernatants were filtered with 0.22 µm filters (VWR, Radnor, PA, USA). Then, the CFSs were stored at 4 °C [24,25].

2.1.1. Antimicrobial Activity of the CFS by Agar Well Diffusion Assay

Nine micro-organisms, both pathogenic and spoiling, including different bacteria and yeasts, were selected as indicator micro-organisms (Table S3). In the case of pathogenic micro-organisms, the decision was taken based on the information collected in Rapid Alert System Feed and Food on the main pathogens responsible for food alerts in Europe in 2023 [4]. Spoilage micro-organisms were chosen among the most common micro-organisms in fresh and minimally processed foods, according to CNTA experience.

The antimicrobial activity of the potential CFSs against the indicator strains was determined by using the modified agar well diffusion assay [26]. Briefly, TSA-YE agar (0.8%) was autoclaved, poured 20 mL onto each petri dish (VWR) of 90 mm, and solidified. When the plates were ready, a concentration of 10^6 CFU/mL of the indicator micro-organism was evenly added and spread with a sterile Digralsky loop to form lawn growth cells. Then, wells of 7.2 mm in diameter were made in the plates using a sterile borer, and the wells were filled with 100 µL of the testing CFS (Table S1). The plates were kept for 2 h at room temperature on the bench to aid the diffusion. Finally, they were incubated in aerobic or anaerobic conditions at different temperatures for 24 or 48 h depending on the micro-organism tested. After that time, the inhibition halo was measured. Considering the criteria outlined by Lesteri et al. [27] and Imade et al. [28], the antimicrobial activity of the CFSs was classified based on the inhibition halo showed by indicator micro-organisms when exposed to each CFS. Including the diameter of the well (7.20 mm), it was deemed negative when the resulting halo was less than 9.20 mm above the diameter of the well, while a halo ranging from 9.20 to 15.0 mm was considered medium activity. If the inhibition halo ranged from 15.00 mm to 25.00 mm, it was categorized as high antimicrobial activity. A halo of more than 25.0 mm was considered as strong antimicrobial activity.

2.1.2. Minimum Inhibitory Concentration (MIC)

To further characterize the antimicrobial activity exhibited by CFS of the selected strains, and to know the dynamic interaction and the antimicrobial activity over time of each of the CFS against the indicator micro-organisms *L. monocytogenes* and *L. parabuchneri*, the time–kill curve was determined, by which the MICs were also obtained. For this purpose, a sterile 96-well flat-bottom microtiter plate (DELTALAB) with increasing concentrations under 10% of the CFSs (0%, 0.15%, 0.31%, 0.625%, 1.25%, 2.5%, and 5%) were used which were confronted to a final well concentration of 10^5 CFU/mL of the indicator micro-organism, either *L. monocytogenes* or *L. parabuchneri*. The plates were incubated at the optimum growth conditions of the target micro-organism and were monitored visually and using a plate reader (mod. EPOCH 2 NS, Agilent, Santa Clara, CA, USA) at a wavelength of 620 nm every 2 h. Finally, the MIC was established as the minimum concentration of CFS necessary to inhibit the growth of the target micro-organism.

2.2. In Vitro Determination of Antibiotic Resistance

The antibiotic resistance of selected isolates was determined by the modified ISO 10932/IDF 223 standard [29] for the Determination of the Minimal Inhibitory Concentration (MIC) of antibiotics applicable to bifidobacterial and non-enterococcal LAB using the microdilution method. MICs were determined for nine antibiotics: kanamycin (Acros Or-

ganics Bvba, Geel, Belgium), gentamicin, streptomycin, tetracycline, clindamycin, chloramphenicol, ampicillin, erythromycin (Merck KGaA, Darmstadt, Germany), and vancomycin (Merck KGaA). The MICs were assessed as the lowest concentration of the antibiotic that inhibited bacterial growth. Based on the MICs, strains were classified as either resistant or susceptible, adhering to the cut-off values and guidelines established by the FEEDAP [30] for LAB and by the SCAN [31] for the *Bacillus* strain.

The values were calculated from three independent experiments and were classified as susceptible (S) when the result was under the cut-off value or resistant (R) when the value was above the cut-off value phenotypes.

2.3. Evaluation of the Cytotoxicity of the Cell-Free Supernatant of B. subtilis CNTA 517 on Epithelial Cells

In vitro assessments of the cytotoxicity of the CFS of *B. subtilis* were conducted using Caco-2 and HT-29 cell lines; both were human colorectal cancer cells and were obtained from the Leibniz Institute DSMZ—German Collection of Micro-organisms and Cell Cultures GmbH (DSMZ) (Braunschweig, Germany) collection.

The cytotoxicity was determined by the MTT method (3- (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Merck KGaA). The cell lines were seeded in a 96-well flat-bottom microtiter plate at varying concentrations: 5×10^3 cells/well for the Caco-2 cell line and 1.7×10^4 cells/well for the HT-29 cell line. Subsequently, they were incubated at 37 °C, 5% CO_2, and 95% relative humidity for 48 h in the CO_2 incubator SCA-165DRS (ASTEC Co., Ltd., Fukuoka, Japan). In the case of the Caco-2 cell line, the medium was refreshed three times per week. After 15 days of growth to allow cell differentiation into enterocytes, *B. subtilis* CFS was introduced at varying concentrations (5% and 20%). For the HT-29 cell line, the concentrations of 5%, 10%, and 20% of *B. subtilis* CFS were added after 48 h of incubation. The entire volume of cell supernatants was transferred to 96-well U-bottom microtiter plates for subsequent analysis and stored at −80 °C. Afterward, the culture medium was removed and 200 µL of phenol red-free medium (Thermo Fisher Scientific, Waltham, MA, USA) and 50 µL of MTT at a concentration of 5 mg/mL to each well were added. These plates were then placed at 37 °C, 5% CO_2, and 95% relative humidity in the incubator for 3 h until the observation of thread formation under the inverted microscope ECLIPSE TS100/TS100F (Nikon MicroscopyU, Melville, NY, USA). When the established time had elapsed, the culture medium was removed to eliminate unmetabolized MTT. Finally, 150 µL/well of dimethyl sulfoxide (DMSO) (Thermo Fisher Scientific) was added and agitated at 347.76 g for 5 min to dissolve the formazan deposits produced. Then, 100 µL of the supernatant was transferred to a new 96-well flat-bottom plate (DELTALAB, Barcelona, Spain), and the optical density (OD) was measured at a wavelength of 570 nm using the plate reader HEALES, MB-580 (Shenzhen Heales Technology Development Co., Ltd., Shenzhen, China). The cytotoxicity of the CFS of the strain *B. subtilis* CNTA 517 was measured by the viability of the cell lines used following Equation (1):

$$\%\ viability = \frac{OD570\ sample}{OD570\ control} \times 100 \quad (1)$$

With this percentage as a basis, statistical significance studies were conducted to ascertain whether there was a decrease in cell cytotoxicity or not.

2.4. Investigation of the Nature of the Antimicrobial Compounds

The antimicrobial activity shown by CFSs could be attributed to various compounds released by the potential protective culture bacteria. Among these, organic acids, hydrogen peroxide, and peptides are commonly the most relevant. To assess whether the antimicrobial activity of the CFSs was due to one or another compound, some assays were carried out in which the indicator micro-organisms were *L. monocytogenes* and *L. parabuchneri*.

2.4.1. Synthesis of Organic Acids and Their Contribution to Antimicrobial Activity

To ascertain the concentration of organic acids (lactic and acetic) and phenylactic acid produced by the selected strains, HPLC was employed. This allowed us to identify the strain that exhibits higher production of either acid under the specified growth. Additionally, to elucidate the influence of the acids on the antimicrobial activity, the stability of the CFSs at different pH levels was determined.

Organic Acids Concentration by HPLC

The concentrations of lactic acid and acetic acid in the samples were quantified using the e2695 high-performance liquid chromatography (HPLC) system (Waters Alliance, Framingham, MA, USA), which was equipped with an Aminex Column HPX-87H 300 × 7.8 mm (BIO RAD, Berkley, MI, USA) along with its precolumn Micro-Guard Cation H refill cartridges, 30 × 4.6 m (BIO RAD). The run time was 30 min per sample with an injected volume of 10 µL. The mobile phase used was 0.005 M H_2SO_4 at a flow rate of 0.6 mL per min. The retention times were 12.564 min for lactic acid and 14.783 min for acetic acid. Detection was carried out using the visible ultraviolet detector PDA 210 nm (2996) (Waters Alliance), following the internal standard method.

In the case of phenylactic acid, its concentration was specifically determined using the method outlined by Zheng et al. [32]. The HPLC system employed remained consistent with the previously mentioned e2695 system (Waters Alliance), while the chromatographic column used was CORTECS C18 2.7 micron (100 × 4.6 mm) (Waters Alliance). The retention time was 6.20–6.40 min.

Two measurements of each acid concentration were performed on the same day.

Antimicrobial Activity of the Cell-Free Supernatants at Different pHs

To indicate whether the antimicrobial activity was attributable to the presence of organic acids, the pH of the CFS samples was acidified (pH 3.50) and neutralized (pH 7.00), being lower or higher than the pKa of the different expected acids, respectively (pKa of lactic acid at 3.80; pKa of phenylacetic acid at 4.30; and pKa of acetic acid at 4.76). pH adjustments were achieved through the addition of 1 M NaOH or 1 M HCl [33,34]. The antimicrobial activity of the pH-modified CFS against the indicator micro-organisms was determined by the agar well diffusion assay described above, using the untreated CFS as a reference.

If the halo disappeared when neutralizing the CFS, it suggested that the antimicrobial activity was attributed to the organic acids produced by the bacteria. In contrast, if no significant differences were observed ($p > 0.05$), it indicated that the antimicrobial activity of the CFS remained consistent across different pH levels. This suggested that the antimicrobial activity primarily stemmed from the production of a compound other than organic acids [35].

2.4.2. Bacteriocin-like Substance Production by the Cell-Free Supernatants

To confirm that the antimicrobial activity of the potential protective cultures was associated with proteinaceous compounds related to bacteriocin production, the CFSs were subjected to enzymatic treatment using a mixture of two proteases: proteinase K and pepsin (Merck KGaA) [36]. These were simultaneously added; 25 µL of proteinase K was mixed with 5 mL of the CFS, while, in the case of pepsin, a 0.003 M phosphate buffer was prepared in advance, from which 1 mL was used to dissolve 1 mg of pepsin. Subsequently, 50 µL of this pepsin solution was added to 5 mL of the CFS. Finally, the mixture was incubated for 1 h at 37 °C under aerobic conditions and the antimicrobial activity of these protease-treated CFSs was determined by the agar well diffusion method (Section 2.1.1) against the indicator micro-organisms, using as reference the CFS without treatment.

If the halo disappeared after the addition of the protease mix, the production of proteinaceous nature compounds (BLIS or bacteriocin-like substances) could be confirmed. However, if the halo persisted, the nature of the compound remained undetermined. In

such cases, two additional proteases were added to the CFSs at varying concentrations which included protamex (NOVOZYMES, Bagsværd, Denmark) at 0.1, 0.5, and 1 mg/mL, and pancreatin (Merck KGaA) [36] at the same concentration range. These were confronted with the indicator micro-organism using the agar well diffusion assay, and the resulting zone of inhibition was quantified. In cases where no halo was observed, signifying a lack of antimicrobial activity in the CFS, it served as evidence of the proteinaceous nature of the compound. If the halo did not disappear, more assays would be needed.

2.5. Thermal Stability of the Cell-Free Supernatants

The heat resistance of the CFSs was measured to know whether the antimicrobial activity of CFSs would be maintained if they were added to foods that would subsequently receive a pasteurization or sterilization treatment (commonly used by the food industry). These treatments were used as a reference to select the intensity (temperature and time) of the different heat treatments (from 10 to 120 min at 70, 90, 110, and 120 °C).

For low temperatures (70 °C and 90 °C), heat treatments were carried out in a thermoblock (Eppendorf, Hamburg, Germany), and, for high temperatures (110 °C and 121 °C), an oil bath (Leyro Instruments, San Sebastián de los Reyes, Spain) was used. A small portion of CFS was added in preheated liquid to ensure the initial temperature, which was measured by the temperature sensor Checktemp 1 HI98509 (HANNA instruments, Limena, Italia). After the preset intervals, the eppendorfs or vials were removed and introduced in cold water. Afterward, the indicator micro-organisms *L. monocytogenes* and *L. parabuchneri* were exposed to the treated CFSs following the agar well diffusion methodology.

Finally, taking as a reference the halo obtained by agar well diffusion assay (Section 2.1.1) of the untreated CFSs against *L. monocytogenes* and *L. parabuchneri*, a formula (Equation (2)) was applied to determine the percentage of antimicrobial activity maintained by each CFS following exposure to various combinations of temperatures and durations.

$$\%\ loss\ antimicrobial\ activity = \frac{halo\ diameter\ (mm)_{before\ heat\ treatment} - halo\ diameter\ (mm)_{after\ heat\ treatment}}{halo\ diameter\ (mm)_{before\ treatment}} \times 100 \quad (2)$$

Following the representation of the percentage of the retained antimicrobial activity of the CFS throughout time at each temperature, a linear regression was obtained to estimate the loss of antimicrobial activity of the CFS under commercial heat treatments applied by the food industry.

2.6. Whole-Genome Sequencing of the Strains, Identification of AMR Genes, and Identification of Biosynthetic Gene Clusters

The sequenced genome of the *Bacillus subtilis* CNTA 517 strain was obtained through whole-genome sequencing (WGS) using an Illumina iSeq platform (NASERTIC, Pamplona, Spain). Genomic DNA was extracted from 1 mL of fresh culture using the GenElute™ bacterial Genomic DNA kit (Merck KGaA), and tagmented with the Nextera XT DNA library prep kit. The 2 × 150-bp paired-end sequencing reads generated were pre-processed by removing adapters, short reads (<140 bp), and poor quality reads (Phred score < 28) using Trimmomatic v.0.39 [37]. After the quality of the filtered clean reads was checked with FastQC v.0.12.1 [38], de novo genome assembly was performed with Shovill v.1.1.0 [39] using the SPAdes assembler with default parameters. Quast v.5.2.0. [40] was used to evaluate the quality of the assembly. Then, 24 long contigs (>500 base pairs) were generated, with 99.24% of reads successfully mapped to the assembly.

The assembled contigs in FASTA format were screened for antimicrobial resistance genes (ARGs) using the ABRicate v. 1.0.1 tool [41] and the CARD [42,43] and ResFinder [44,45] with a minimum identify and coverage values of 80%. The assembled contigs were additionally screened for mobile genetic elements using the MobileElementFinder v.1.0.3 tool [46,47]. Finally, antiSMASH v.6.1.1. [48] was used to predict the type and location of secondary metabolite biosynthesis gene clusters within the assembled genomes.

2.7. Data Analysis and Representation

NCBI's Taxonomy Browser [49], R [50], RStudio 2023.12.1, and the packages ggplot2 [51] and ggtree [52] were used to visualize the initial screening of all CFSs or the active ones against the selected panel of indicator micro-organisms. GraphPad PRISMA software v. 5.00 was used to represent the rest of the graphs. Statistical analyses for comparing each group of samples with the control group were performed in GraphPad using an independent-sample *t*-test at a significance level of 0.05. Normality was assumed for all samples. Since homogeneity of variances could not be assumed, a Brown–Forsythe ANOVA test with Dunnett's multiple comparisons post hoc tests was applied to compare the lactic acid production of the four tested strains at a significance level of 0.05. The same test was applied to compare the acetic acid production among the LAB strains.

3. Results and Discussion

3.1. Preliminary Selection of Potential Bacterial Strains to Be Protective Cultures and Characterization of Their Antimicrobial Activity

The CFSs of 200 strains of potential protective cultures were subjected to the agar well diffusion assay against the indicator micro-organisms. Figure S1 shows the inhibition zones obtained with CFSs derived from cultures that grew normally. Approximately 60% out of all the CFSs exhibited a certain antimicrobial activity, and those strains are depicted in Figure 1. These selected CFSs were more active against Gram-positive indicator micro-organisms, particularly *L. monocytogenes*, *Bacillus cereus*, and *L. parabuchneri*. The observed inhibition halos ranged from 0.92 to >25 mm. Among all the strains under consideration, three representatives of LAB (*Pediococcus acidilactici* CNTA 1059, *Lactiplantibacillus plantarum* CNTA 600, and *Levilactobacillus brevis* CNTA 1374) and one from *Bacillus* sp. (*Bacillus subtilis* CNTA 517) were chosen due to their heightened antimicrobial activity against *L. monocytogenes* and *L. parabuchneri*, exceeding a 15 mm inhibition halo (Figure 1). These strains were isolated from an asparagus by-product (*P. acidilactici* CNTA 1059), homemade *chorizo* (*L. plantarum* CNTA 600 and *L. brevis* CNTA 1374), and an altered paint (*B. subtilis* CNTA 517).

3.2. Antimicrobial Activity of the CFS

The efficacy of the CFSs produced by the selected strains as a potential protective culture against various species of pathogenic and spoilage micro-organisms is shown in Figure 1 and with more detail in Table S4. In the case of the pathogenic target strains, the resulting inhibition halo for the CFS of the strain *P. acidilactici* CNTA 1059 against *L. monocytogenes* was 24.53 ± 0.21 mm, indicating robust antimicrobial activity. This observation aligns with the results obtained by Hartmann et al. [9] for their strain IDE0550 against three different *L. monocytogenes* strains. Similarly, the CFS of the selected strain *L. plantarum* CNTA 600 exhibited a strong antimicrobial effect against *L. monocytogenes* (15.27 ± 0.47 mm), consistent with the findings of Vataščinová et al. [53]. A medium antimicrobial activity against *B. cereus* (13.36 ± 0.67 mm) was observed, corroborating similar previous results by Akhtar and Syed [54]. *L. brevis* CNTA 1374 CFS showed no antimicrobial activity against any of the pathogenic strains. This contradicted the findings of Jang et al. [55], who reported the antimicrobial activity of the *L. brevis* KU15153 strain against food pathogens, including *L. monocytogenes*, *S. aureus*, *Salmonella* Typhimurium, and *E. coli*. Such discrepancies may be highlighting the variability in antimicrobial activity among different strains of the same species.

Finally, the antimicrobial activity of CFS *B. subtilis* CNTA 517 was strong against *L. monocytogenes* (28.98 ± 0.07 mm) and against *S. aureus* (19.36 ± 0.07 mm). Different authors have reported the strong antimicrobial activity of purified bacteriocins produced by *B. subtilis* against these two pathogenic micro-organisms. Wei et al. [56] documented the antimicrobial activity of subtilosin JS-4, while Taggar et al. [57] described that of peptide-Ba49. However, the reported activity of these peptides was lower than the antimicrobial activity of *B. subtilis* CNTA 517 described in this paper.

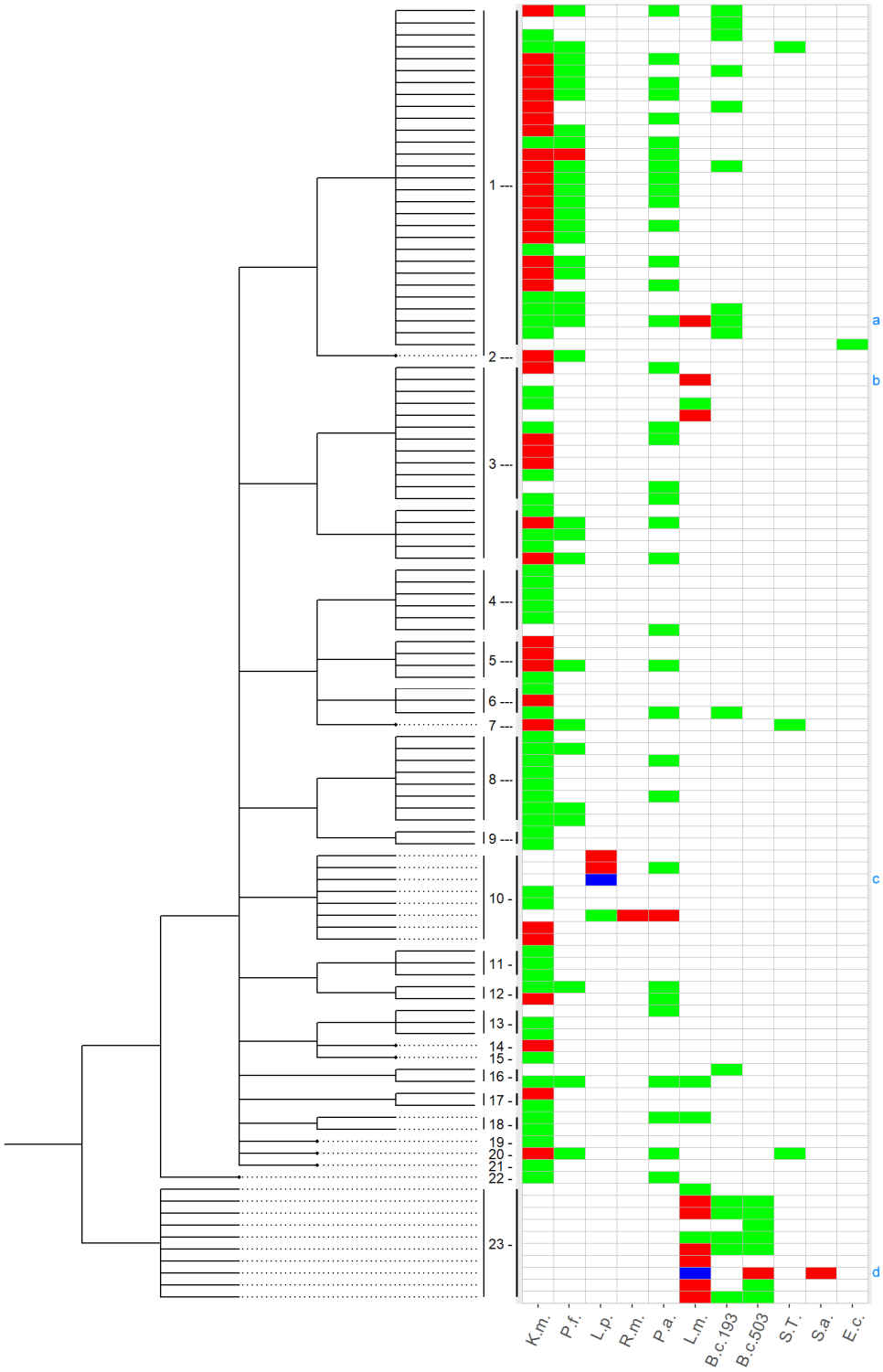

Figure 1. Depiction of the inhibition zone achieved by each selected culture-free supernatant: <9.2 mm (white), 9.2 mm ≤ inhibition zone < 15.0 mm (green), 15.0 mm ≤ inhibition zone < 25.0 mm (red), and ≥25.0 mm (blue). Tested bacterial species: K.m. = *Kluyveromyces marxianus* CNTA.1649, P.f. = *Pseudomonas fluorescens* CNTA 571, L.p. = *Lentilactobacillus parabuchneri* DSMZ 5987, R.m. = *Rothia mucilaginosa* DSMZ 18184, P.a. = *Pseudomonas aeruginosa* CECT 110T, L.m. = *Listeria monocytogenes* CECT 7467, B.c.193 = *Bacillus cereus* CECT 193, B.c.503 = *Bacillus cereus* CNTA 503, S.T. = *Salmonella* Typhimurium CECT 443, S.a. = *Staphylococcus aureus* CECT 976, and E.c. = *Escherichia coli* DSMZ 19206. A dendrogram showing the taxonomic hierarchical relationship between the different tested

strains is also shown. 1 = *Lactiplantibacillus plantarum*, 2 = *Lactiplantibacillus pentosus*, 3 = *Pediococcus acidilactici*, 4 = *Lacticaseibacillus paracasei*, 5 = *Lacticaseibacillus rhamnosus*, 6 = *Lacticaseibacillus casei*, 7 = *Lacticaseibacillus manihotivorans*, 8 = *Limosilactobacillus fermentum*, 9 = *Limosilactobacillus reuteri*, 10 = *Levilactobacillus brevis*, 11 = *Lactobacillus delbrueckii*, 12 = *Lactobacillus johnsonii*, 13 = *Leuconostoc mesenteroides*, 14 = *Leuconostoc citreum*, 15 = *Leuconostoc pseudomenteroides*, 16 = *Latilactobacillus curvatus*, 17 = *Weissella confusa*, 18 = *Companilactobacillus alimentarius*, 19 = *Liquorilactobacillus nagelii*, 20 = *Ligilactobacillus salivarius*, 21 = *Oenococcus oeni*, 22 = *Enterococcus mundtii*, 23 = *Bacillus subtilis*. Selected cultures: a = *Lactiplantibacillus plantarum* CNTA 600, b = *Pediococcus acidilactici* CNTA 1059, c = *Levilactobacillus brevis* CNTA 1374, and d = *Bacillus subtilis* CNTA 517.

The strain *L. monocytogenes* CECT 7467 was selected as the pathogenic indicator micro-organism to be used in the next assays for the CFSs of *P. acidilactici* CNTA 1059, *L. plantarum* CNTA 600, and *B. subtilis* CNTA 517. In addition to its sensitivity to the CFSs of the strains tested, it is a micro-organism capable of growing in refrigeration and whose presence has accounted for 15% of foodborne illnesses according to the annual report of the RASFF of the year 2023 [4].

The antimicrobial activity of the CFSs from *P. acidilactici* and *B. subtilis* species against the selected spoilage micro-organisms could be classified as negative or null, as they did not produce any halos in the agar well diffusion assay. On the contrary, the CFS of *L. plantarum* CNTA 600 exhibited a medium activity against *P. fluorescens* (10.78 $\pm$ 0.05 mm) which aligned with the activity described by Lv et al. [58]. It also demonstrated strong activity against *K. marxianus* (14.14 $\pm$ 0.12 mm), for which the antimicrobial activity of different antimicrobials has not been described in the literature yet [59]. Notably, the CFS displaying the highest antimicrobial activity was the one from *L. brevis* CNTA 1374, producing halos of 32.20 $\pm$ 0.04 mm against the spoilage LAB *L. parabuchneri*, not previously described. Considering these results and its ability to produce histamine in cheese [60], *L. parabuchneri* CNTA 1075 was selected as the spoilage indicator micro-organism to be tested against the CFS of *L. brevis* CNTA 1374 in the following tests.

The MIC of the CFS of the different species was determined by the time–kill curve and was seen to be specific for each strain but within a similar range (Figure S2). The MICs of *P. acidilactici* CNTA 1059, *L. plantarum* CNTA 600, and *B. subtilis* CNTA 517 against *L. monocytogenes* were 2.5%, 5.0%, and 1.25%, respectively. In the case of *L. brevis* CNTA 1374, a lower MIC of 0.15% was determined against *L. parabuchneri*. These results align perfectly with those obtained with the agar diffusion assay, which showed *L. brevis* CNTA 1374 to attain the greatest inhibition halo, while *L. plantarum* CNTA 600 showed the weakest activity out of all the selected strains.

3.3. Antibiotic Resistance of the Selected Strains

The MIC was determined by a broth microdilution assay and the results in Table 1 indicated that, among LAB, only *L. brevis* CNTA 1374 demonstrated sensitivity to all antibiotics tested according to the referenced cut-off values [30]. However, it should be noted that kanamycin resistance has been described as intrinsic in pediococci and most lactobacilli species (including *L. plantarum*) [61–63]. This has been observed in numerous previous works and has been explained by the high rate of spontaneous chromosomal mutations conveying resistance to aminoglycosides, having a low potential for horizontal spread [61,64,65].

In the case of *B. subtilis* CNTA 517, Adimpong et al. [66] already pointed out that the cut-off antibiotic values defined by SCAN [31] more closely reflect the intrinsic resistances of *B. subtilis*, in contrast to those set by FEEDAP [30]. In this regard, *B. subtilis* CNTA 517 was deemed susceptible to all tested antibiotics. Furthermore, no mobile genetic elements were found after sequencing this strain, and all the detected antibiotic resistance genes (detailed in Table S5) are also found in the type of strain *B. subtilis* subsp. *subtilis* str. 168 (NCBI Reference Sequence NC_000964.3). These findings support the hypothesis that *B. subtilis*

CNTA 517, like the three tested LAB strains, does not harbor any acquired antimicrobial resistance which could be transferred to other bacteria.

Table 1. Antibiotic resistance of the selected strains according to the broth microdilution assay. The S/R values were taken following the FEEDAP cut-off values for *Pediococcus* spp., *Lactobacillus plantarum/pentosus*, *Lactobacillus* obligate heterofermentatives [30], and SCAN values for the *Bacillus* sp. [31].

Potential Protective Culture	Antibiotic Resistance *								
	Resistant/Sensitive								
	GEN	KAN	STR	TET	CLN	CHL	AMP	ERY	VAN
Pediocccus acidilactici CNTA 1059	S	R	S	S	S	S	S	S	n.r.
Lactiplantibacillus plantarum CNTA 600	S	R	n.r.	S	S	S	S	S	n.r.
Levilactobacillus brevis CNTA 1374	S	S	S	S	S	S	S	S	n.r.
Bacillus subtilis CNTA 517	S	S	R	S	S	S	n.r.	S	S

* GEN: gentamycin; KAN: Kanamycin; STR: Streptomycin; TET: Tetracycline; CLN: clindamycin; CHL: cloramphenicol; AMP: ampicilin; ERY: erytromycin; VAN: vancomycin. S: sensitive to antibiotic; R: antibiotic resistant; n.r.: not required.

3.4. Evaluation of the Cytotoxicity of the Cell-Free Supernatant of B. subtilis CNTA 517 on Epithelial Cells

The effect of different concentrations of the CFS of *B. subtilis* CNTA 517 on Caco-2 and HT-29 (Figure S3) cell lines was determined by the MTT method. At the assayed conditions, the concentrations of the tested CFSs were found to be non-toxic. These results coincide with the research presented in the works of Brutscher et al. [67] and Chen et al. [68], affirming the non-toxigenic characteristics of this micro-organism in epithelial cells. According to these observations, the CFS of the strain *B. subtilis* CNTA 517 could, therefore, be considered as an ingredient for industrial use in food.

3.5. Nature of the Antimicrobial Compounds Contained in CFS

The nature of the antimicrobial compounds produced by the bacteria could be of organic origin, so the organic acid-producing capacity of the selected strains was determined. On the other hand, the change in pH of the CFS was related to the antimicrobial activity they showed, and it was possible to determine whether it was due to the production of these compounds or to the production of antimicrobial peptides.

3.5.1. The Synthesis of Organic Acids and Their Contribution to Antimicrobial Activity

In a first step, the concentration of organic acids was measured by HPLC. Lactic acid and acetic acid stand out as the two most prevalent organic acids generated by LAB [69]. Table 2 shows the concentration of the organic acids of each CFS. Concerning lactic acid, *P. acidilactici* CNTA 1059 exhibited the highest production yielding 8.20 ± 0.19 g/L, similar to the previously described for another *P. acidilactici* strain [70]. In contrast, *B. subtilis* CNTA 517 demonstrated the lowest lactic acid production, reaching a value of 2.70 ± 0.17 g/L, which is lower than the 3.63 g/L observed for other *B. subtilis* strains [71].

Table 2. Organic acids concentration on the cell-free supernatants (CFSs). Different superscripts indicate statistical differences among the mean lactic acid or acetic acid production of the tested strains.

Strain	Organic Acids (g/L)		
	Lactic Acid	Acetic Acid	Phenylactic Acid
Pediococcus acidilactici CNTA 1059	$8.20^{a} \pm 0.19$	$3.58^{x} \pm 0.07$	119.13 ± 1.17
Lactiplantibacillus plantarum CNTA 600	$7.39^{a} \pm 0.13$	$3.64^{x} \pm 0.00$	-
Levilactobacillus brevis CNTA 1374	$2.80^{b} \pm 0.02$	$3.78^{x} \pm 0.07$	-
Bacillus subtilis CNTA 517	$2.70^{b} \pm 0.17$	<0.25	-

The maximum production of acetic acid was observed by strain *L. brevis* CNTA 1374 amounting up to 3.78 ± 0.07 g/L, surpassing the levels reported by some authors [72]. Notably, *B. subtilis* CNTA 517 did not exhibit detectable levels. Lastly, *P. acidilactici* CNTA 1059 demonstrated a high production capacity, yielding 119.13 ± 1.17 mg/L, which was aligned with the production described by Bustos et al. [70] of 117.7 ± 1.25 mg/L.

Secondly, the pH of the CFSs was modified to elucidate whether the measured organic acids were partially responsible for the antimicrobial activity. Table 3 shows the native pHs of the tested CFS, alongside the antimicrobial activity of the CFS of the four microorganisms selected under pH 3.50 and 7.00. Acidifying or neutralizing the CFS of strain *P. acidilactici* CNTA 1059 did not affect the native antimicrobial activity (24.53 mm). This aligned with the results of Liu et al. [73] regarding the stability of the antimicrobial activity of another *P. acidilactici* strain. Similarly, the CFS of *L. brevis* CNTA 1374 also showed stable antimicrobial activity (compared to the original 32.20 mm of halo size) when its pH was modified, consistent with findings by Sreedharan et al. [74]. In contrast, a significant decrease in antimicrobial activity (from 22.35 mm of original halo size) was observed when acidifying or neutralizing the CFS of *L. plantarum* CNTA 600. This agrees with the results reported by Akhtar and Syed [54] for a *L. plantarum* strain isolated from yogurt. The CFS from *B. subtilis* significantly decreased its native antimicrobial activity (28.52 mm) under acidic conditions, similar to what was previously observed by Epparti et al. [75]. From these results, it could be inferred that the antimicrobial activity of CFSs is not exclusively due to the production of organic acids, and further research is needed.

Table 3. Antimicrobial activity of the cell-free supernatants (CFSs) at pH 3.50 and 7.00.

Strain	CFS pH	Halo Diameter (mm)	
		CFS pH 3.50	CFS pH 7.00
Pediococcus acidilactici CNTA 1059	4.33	23.27 ± 0.72	23.10 ± 0.56
Lactiplantibacillus plantarum CNTA 600	4.44	20.14 * ± 0.95	19.32 * ± 0.26
Levilactobacillus brevis CNTA 1374	5.06	31.89 ± 0.46	31.52 ± 0.12
Bacillus subtilis CNTA 517	6.77	14.93 * ± 0.01	28.98 ± 0.08

* Significantly different from native pH halo size ($p \leq 0.05$).

3.5.2. Antimicrobial Peptide Production by the Cell-Free Supernatants

The antimicrobial activity of the CFS after a protease treatment with proteinase K and pepsin was performed by the agar well diffusion assay against *L. monocytogenes* and *L. parabuchneri*. The original antimicrobial activity achieved with the CFSs of the three tested LAB strains was absent when the CFSs were treated with proteases. This suggests that the antimicrobial activity of these three strains is likely attributed to their ability to produce bacteriocins, as observed for other strains of these species. The ability of *P. acidilactici* to produce bacteriocins was supported by the findings of Fugaban et al. [76] that described the production of pediocin PA-1 in both *P. acidilactici* and *P. pentosaceus*. Similarly, Wu et al. [77] substantiated the bacteriocin-producing capacity of *L. plantarum* RUB 1 via bacteriocin-synthesis-related genes, while Thapar et al. [78] and Sreedharan et al. [74] supported the BLIS production in *L. brevis*.

The protease-treated CFS of the *B. subtilis* strain CNTA 517 achieved an inhibition zone diameter of 26.40 ± 0.69 mm, indicating no statistically significant differences ($p > 0.05$) compared to the untreated CFS. A subsequent proteolytic step was conducted on the *B. subtilis* CFS with the enzyme pancreatin and the commercially available protease mix PROTAMEX, but no further reduction of the inhibition zone diameter was achieved. Although *B. subtilis* is known to produce over 20 bacteriocins with distinct functional and structural properties [79], in this case, the nature of the antimicrobial activity remained undetermined, and, therefore, the WGS of this strain was performed.

3.5.3. Antimicrobial Compounds Produced by *B. subtilis* Strain CNTA 517

To help elucidate the antimicrobial activity exhibited by *B. subtilis* CNTA 517, gene cluster mining was carried out on its WGS. Table 4 shows the result of the AntiSMASH screening.

Table 4. Identified secondary metabolite regions in the genome (assembled contigs) of *Bacillus subtilis* CNTA 517 using AntiSMASH with strictness 'relaxed'.

Region (contig:from-to)	Type	Most Similar Known Cluster	Similarity
01:93826-115524	epipeptide	thailanstatin A	10%
01:335810-377228	other	bacilysin	100%
01:380214-401825	sactipeptide	subtilosin A	100%
01:618860-639606	CDPS	pulcherriminic acid	100%
01:922093-973870	NRP-metallophore, NRPS	bacillibactin	100%
02:317794-358891	T3PKS	1-carbapen-2-em-3-carboxylic acid	16%
02:407348-429246	terpene		
02:502119-579867	NRPS, betalactone	fengycin	100%
02:644080-758852	transAT-PKS, NRPS, T3PKS, PKS-like	bacillaene	100%
03:1-21708	NRPS	-	-
03:430506-451309	terpene	-	-
05:199657-265045	NRPS	surfactin	78%
05:395661-418614	sactipeptide, ranthipeptide	sporulation killing factor	100%
10:1-6820	NRPS	pelgipeptin	37%
14:1-1179	NRPS		

This analysis uncovered the presence of gene clusters responsible for the synthesis of several antimicrobial compounds produced by *B. subtilis*: polyketides (subtilosin, bacillaene), the lipopeptide fengycin, the catechol siderophore bacillibactin, and the dipeptide bacilysin. Gene clusters with a lower similarity to known clusters involved in the biosynthesis of the lipopeptides surfactin and pelgipeptin, as well as two unidentified gene clusters coding for non-ribosomal peptides, were also annotated. With the exception of pelgipeptin, all these compounds have previously been reported to be synthesized by diverse *B. subtilis* strains [80]. *B. subtilis*'s lipopeptides have shown enhanced resistance to enzyme proteolysis and stable activity within a pH range of 4.0–12.0 but reduced activity at lower pH [81]. Considering the results obtained with the CFS of *B. subtilis* CNTA 517, the antimicrobial activity of this strain may be attributed, among others, to the production of surfactin and/or a pelgipeptin-like compound. On the other hand, it should be noted that *B. subtilis* CNTA 517 exerted a superior antimicrobial activity when compared to the rest of the tested *B. subtilis* strains (Figure 1). Consequently, the isolation of the pelgipeptin-like compounds and the other two putative peptides for their further characterization would be of great interest to advance the study of bioprotective agents.

3.6. Thermal Stability of the Cell-Free Supernatants

To assess the suitability of these CFSs for application in food products, different pasteurization and sterilization treatments were explored.

Examples of pasteurization treatments are 72 °C/15 s [82] for milk pasteurization or 90–95 °C for 15–30 s (High Temperature Short Time—HTST), a common treatment applied to the stabilization of citric juices [83]. Pasteurization treatments assayed at 70 and 90 °C for even 90 min did not induce a significant ($p > 0.05$) reduction in the antimicrobial activity of the tested CFSs. Therefore, all the CFSs of the four protective cultures could be considered suitable to be added to products subjected to any kind of pasteurization treatment during their production.

In the case of industrial sterilization treatments, three different practices were selected as examples to apply the equations obtained from the laboratory treatments: an in-container sterilization of milk at 110 °C for 20 min, 3 min botulinum cooking at 121 °C (commonly applied to canned food), and an autoclaving processing of 15 min at 121 °C (commonly

applied to some materials in contact with food). Treatments at 110 and 121 °C for 90 min did result in some loss of activity, depending on both the specific treatment and the tested CFS (Table S6). For the in-container sterilization, the estimated loss of antimicrobial activity would range between 8 and 21%, maintaining 79–92% of the activity. For the botulinum cooking, it would result in a loss of activity ranging from 3% to 13%, so 87–97% of the antimicrobial activity would be retained. When the CFSs were autoclaved, the loss of activity ranged between 9 and 28% (Table 5).

Table 5. Estimation of the lost antimicrobial activity of the cell-free supernatants (CFSs) after two examples of commercial sterilization treatments.

Strain	% of Antimicrobial Activity Lost after the In-Container Milk Sterilization Treatment (110 °C/20 min)	% of Antimicrobial Activity Lost after Botulinum Cooking (121 °C/3 min)	% of Antimicrobial Activity Lost after Autoclaved Treatment (121 °C/15 min)
Pediococcus acidilactici CNTA 1059	8.18	3.73	11.06
Lactiplantibacillus plantarum CNTA 600	13.92	13.12	22.17
Levilactobacillus brevis CNTA 1374	8.36	4.73	9.02
Bacillus subtilis CNTA 517	21.67	9.44	28.70

These results confirmed the findings from various authors regarding the thermal stability of the CFSs (or BLIS) of the bacteria when treated at 100 °C. However, they diverged when subjected to treatments at 121 °C. Both Dhanda et al. [84] and Sidek et al. [85] reported no antimicrobial activity after the treatment at 121 °C. This contrasts with what was obtained with the CFS of *P. acidilactici* strain CNTA 1059. The results of the thermal stability of CFS *L. plantarum* CNTA 600 aligned with Zangeneh et al. [86], who described as thermostable the bacteriocin produced by a strain isolated from traditional sourdough even after treatment at 121 °C for 15 min. However, Qadi et al. [87] reported the loss of antimicrobial activity of two *L. plantarum* strains after treatment at 121 °C for 15 min. The thermostability of the strain *L. brevis* CNTA 1374 at 121 °C was not supported by Qadi et al. [87], who did not describe any antimicrobial activity after a treatment of 121 °C for 15 min of another strain of L. brevis. Lastly, Hyun et al. [88] described the thermal stability of the BLIS produced by strain B. subtilis BSC35 after a heat treatment of 121 °C for 15 min against *C. perfringens*, and Wei et al. [56] highlighted the stability of the activity exhibited by the bacteriocin subtilin JS-4 from *B. subtilis*.

As all CFSs obtained in this study seem suitable for food subjected to any kind of sterilization treatments, the choice among them will depend on the contaminating microbiota (due to their different antimicrobial spectrum), and the properties of the food itself (like pH or sensory characteristics).

4. Conclusions

Based on the *in vitro* tests conducted, the strains *P. acidilactici* CNTA 1059, *L. plantarum* CNTA 600, *L. brevis* CNTA 1374, and *B. subtilis* CNTA 517 seem to fulfil the necessary requirements to be used as protective cultures. These strains are properly identified to the species level, are listed in the Qualified Presumption of Safety (QPS) list, do not exhibit acquired resistance to target antibiotics, and do not display toxigenicity upon contact with human epithelial cells.

Instead of using the protective cultures, this study proposes the use of the CFS, showing their potential as biopreservatives in the food industry. The antimicrobial spectrum exhibited by the cell-free supernatants of the selected strains was primarily directed against Gram-positive bacteria, encompassing both pathogenic (*L. monocytogenes*) and spoilage (*L. parabuchneri*) micro-organisms.

The observed antimicrobial activity in all cases can be attributed to the production of bacteriocin-like inhibition substances (BLISs) (and/or lipopeptides in the specific case of *B. subtilis* CNTA 517). These compounds demonstrate stability across various pH levels and temperature fluctuations, making them amenable to be used in products subjected

to thermal preservation treatment, even at sterilization temperatures. Nevertheless, it is essential that we note that these results solely depict the *in vitro* capacity, with the subsequent step involving the testing of these CFSs in diverse food matrices to extend shelf life and potentially reduce or eliminate the need for additives or intense treatments. Therefore, further research is imperative to position CFSs as a viable clean-label alternative for food companies.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/app14104016/s1, Figure S1: Depiction of the inhibition zone achieved by each tested culture-free supernatant; Figure S2: Growth over time of the selected indicator micro-organisms in presence of the CFS; Figure S3: The cytotoxic effect of *Bacillus subtilis* CNTA 517 cell-free supernatant (CFS); Table S1: Potential protective culture species from CNTA collection employed for the preliminary screening; Table S2: Potential protective culture micro-organisms' growth conditions; Table S3: Pathogenic and spoilage micro-organisms chosen as indicator strains and growth conditions; Table S4: Antimicrobial activity of the cell-free supernatants (CFSs) against pathogenic and spoilage strains with a well diameter of 7.20 mm; Table S5. Antibiotic resistance genes identified in *Bacillus subtilis* CNTA 517; Table S6: Antimicrobial activity of the cell-free supernatants (CFSs) after a heat treatment of 110 °C and 121 °C at different times; Table S7. Lost antimicrobial activity of the cell-free supernatants (CFSs) after heat treatments at 110 and 121 °C.

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