applied sciences

MDPI

Article

Antimicrobial Activity and Biodiversity Study of a Homemade Vegetable Puree Treated with Antimicrobials from *Paenibacillus dendritiformis*

Laura Mena, María José Grande and Antonio Gálvez *

Department of Health Sciences, University of Jaen, 23071 Jaen, Spain; lmena@ujaen.es (L.M.); mjgrande@ujaen.es (M.J.G.)
* Correspondence: agalvez@ujaen.es; Tel.: +34-953-212160

Featured Application: Strain UJA2219 shows antibacterial activity in a model food system, inducing changes in the food microbiota. This opens the way for future studies on the effects of preparations derived from strain UJA2219 in food preservation, either by direct application in other food systems or for the development of active coatings aimed at controlling food spoilage or pathogenic bacteria.

Abstract: *Paenibacillus dendritiformis* UJA2219 isolated from carrot produces broad-spectrum antibacterial activity. The aim of the present study was to determine the impact of partially-purified cell-culture extracts of strain UJA2219 on the microbial load and bacterial diversity of a homemade vegetable puree. The puree was challenged with an overnight culture of strain UJA2219 or with cultured broth extracts partially purified by cation exchange (CE) chromatography or reversed-phase (RP) chromatography and incubated for 7 days at temperatures of 4 °C or 25 °C. The best results were obtained at 25 °C with the RP extract, decreasing counts of the presumptive *Enterobacteriaceae* below detectable levels. The bacterial diversity of control and treated puree was studied by Illumina paired-end sequencing, using DNA extracted from the puree samples incubated at 25 °C for 24 h. The controls and the puree inoculated with the UJA2219 strain showed an almost-identical bacterial diversity profile, with *Pseudomonadota* (mainly Fam. *Pseudomonadaceae* -gen. *Pseudomonas*- and *Enterobacteriace* as the most abundant groups). The greatest differences in bacterial diversity were obtained in the puree treated with RP extract, showing a decrease in the relative abundance of *Pseudomonadota* (especially gen. *Pseudomonas*) and an increase of *Bacillota* (mainly of the genera *Bacillus*, *Enterococcus* and *Lactococcus*). Results from the study suggest that the antimicrobial preparations from strain UJA2219 have a potential for application in food biopreservation.

Keywords: food preservation; biodiversity; antimicrobial; *Paenibacillus dendritiformis*

Citation: Mena, L.; Grande, M.J.; Gálvez, A. Antimicrobial Activity and Biodiversity Study of a Homemade Vegetable Puree Treated with Antimicrobials from *Paenibacillus dendritiformis*. *Appl. Sci.* **2023**, *13*, 6901. https://doi.org/10.3390/app13126901

Academic Editor: Ramona Iseppi

Received: 30 April 2023
Revised: 5 June 2023
Accepted: 5 June 2023
Published: 7 June 2023

1. Introduction

A puree is a tasty and nutritious food that can have various textures, from very light to thicker [1]. It can be prepared from multiple combinations of different fruits, vegetables, tubers, bulbs, legumes and other herbal extracts [2]. There are many bacteria that can contaminate these purees due to not thoroughly washing the leaves of the vegetables, from enterobacteria to spore-forming bacteria [3]. Endospore-forming bacteria are a concern in food spoilage, especially for cooked and refrigerated foods, such as purees. In commercial purées, several *Bacillus* species have been identified and have been shown to cause food spoilage during storage at abuse temperatures [4]. *Bacillus cereus* is the most important cause of food poisoning [5]. The species *Bacillus licheniformis*, *Bacillus amyloliquefaciens* and *Bacillus subtilis* have also been linked to incidents of foodborne illness [6,7]. One of the current approaches under study to control food spoilage is based on the use of natural antimicrobial substances, such as bacteriocins [8].

Bacteriocins (mainly those produced by lactic acid bacteria) have been widely investigated for food preservation singly or in combination with other hurdles [9–12]. Bacteriocins fall in the category of peptides derived from ribosomal synthesis or ribosomal peptides (RPs). Endospore-forming bacteria produce a wide array of antimicrobial peptides, including RPs and non-ribosomal peptides (NRPs), many of them with complex modifications, such as lipopeptides [13]. Bacteriocins and NRPs from endospore-forming bacteria have an enormous potential for application as antimicrobials, yet only a limited number of studies have addressed their effects in food preservation [14–17].

Paenibacillus dendritiformis is a Gram-positive microorganism, which lives in the soil, forms spores and has the ability to change between different morphotypes [18]. It has been found that when sister colonies of *P. dendritiformis* are cultivated in a medium low in nutrients, they inhibit each other's growth through the secretion of a lethal factor [19]. This fact occurs in quite a few bacteria, and during starvation, competing bacteria within the same colony can lyse their sisters and use them as a source of nutrients [20]. The antimicrobial compounds secreted during bacterial cannibalism are called bacteriocins and generally have a narrow spectrum of activity, as they have to kill only closely related bacteria which are competing for the same resources [21]. Members of the genus *Paenibacillus* can produce diverse antimicrobial peptides, including bacteriocins (such as lantibiotics and pediocin-like bacteriocins), lasso peptides and NRPs (including cationic and non-cationic lipopeptides) [22–24] of *P. dendritiformis*, producing antimicrobial substances have been studied in crop protection and growth promotion [25–27].

Antimicrobial substances from *Paenibacillus* could have potential application in food biopreservation to avoid the deterioration of these foods, both from similar bacteria and from Gram-negative bacteria. The strain *P. dendritiformis* UJA2219 was isolated from fresh carrot as a producer of antimicrobial activity against bacteria of concern in foods, including *Escherichia coli* and *Salmonella enterica*. The genome of strain UJA2219 has been sequenced, and the genome data strongly suggest that this strain may produce a variant of the lasso peptide paeninodin and also carry genetic determinants related to other putative antimicrobial peptides, such as paenibacterin, pelgipectin and paenilamicin (Mena et al., manuscript in preparation). To the best of our knowledge, there are no previous studies addressing the possible application of *P. dendritiformis* for the preservation of vegetable foods. The aim of the present study was to determine the effect of partially-purified extracts from *P. dendritiformis* UJA2219 on a vegetable puree (selected as a model food system) on the total microbial load and on the bacterial diversity of the puree.

2. Materials and Methods

2.1. Preparation of Partially-Purified Extracts

An overnight culture of *P. dendritiformis* UJA2219 grown in brain–heart infusion broth (BHI, Scharlab, Barcelona, Spain) at 30 °C with shaking was inoculated (1% wt/vol) in 2 L of complex medium CM [28] without casamino acids. After 24 h incubation at 30 °C with shaking, the pH was measured and the entire culture was filtered through a glass funnel with Whatman filter paper. The culture filtrate was divided into two aliquots (1 L each) and mixed in bulk with the corresponding chromatography gels for 45 min under shaking at room temperature. The chromatography gels used for the recovery of antimicrobial substances from the culture filtrates were cation exchange Carboxymethyl-Sephadex C-25 (Sigma-Aldrich, Madrid, Spain) and reversed-phase Waters PREP C18 55-105UM gel (Waters, Milford, MA, USA). The gels were then washed with 50 mL distilled water. Eluents (45 mL each) were 1.5 M NaCl in distilled water (for the cation exchange gel) and 40% acetonitrile/water and 60% acetonitrile/water for the reverse-phase gel. Eluates were tested for antibacterial activity by the agar-well diffusion method with Oxford towers (8 mm diameter, Scharlab) according to Tagg and McGiven, 1971 [29]. The bacteria used for sensitivity tests were from our laboratory collection or from the Spanish Type Culture Collection (CECT, Burjasot, Valencia, Spain): *Escherichia coli* E19, *Staphylococcus aureus* CECT

976, *Enterococcus faecalis* S-47, *Listeria innocua* CECT 910, *Salmonella enterica* CECT 3197 and *Bacillus cereus* LWL1.

2.2. Preparation of the Puree

A homemade vegetable puree was prepared by mixing the raw ingredients with skin and without washing and passing the mixture through a blender (Oster. All Metal Drive; Sunbeam Products Inc., Boca Raton, FL, USA). The following ingredients were used (wt/wt): vegetables 90% (vegetables in variable proportion: potato, carrot, leek, zucchini, green beans, chard, cabbage, spinach, peas), olive oil, salt and water.

2.3. Preparation of Puree Samples for Microbiological Counts

The puree was distributed into two sets of 4 Falcon test tubes with 5 mL of puree each (8 tubes in total). The first tube of the set was used as a control (without any addition of antimicrobials). The second tube was inoculated (1% vol/vol) with an overnight culture of *P. dendritiformis* strain UJA2219 in order to test the potential of the bacterium for direct antagonism in the puree. The third tube we added 500 µL of the partially purified with NaCl eluate (E) and the last sample was added 500 µL of the partially purified eluate obtained with 60% acetonitrile (A). One set of tubes was incubated at 4 °C and the other at 25 °C. At desired times of incubation (0 h, 24 h, 48 h and 7 days) the puree samples were tested for viable cell counts. Briefly, one aliquot of the puree (1 mL) was serially diluted in sterile saline solution and plated in triplicate on the culture media Tryptic soy agar (TSA), Eosin Methylene Blue Agar (EMB), *Bacillus cereus* agar and de Man, Rogosa and Sharpe agar (MRS). Viable cell counts were determined after 24–48 h of incubation at 37 °C and 30 °C for MRS medium. All culture media were provided by Scharlab (Barcelona, Spain).

2.4. Preparation of Puree Samples for Biodiversity Analysis

The puree, prepared as described above, was clarified through a strainer in order to minimize the interference of solids with the process of DNA extraction. Then, the puree was distributed into 12 Falcon tubes (2 mL of puree each). The tubes were grouped in four sets (3 tubes each). The first set was used as a control. The second set was inoculated (1%, vol/vol) with an overnight culture of *P. dendritiformis* UJA2219 as described above. The third set was added with 200 µL of the eluate E (per 2 mL of puree) and the last set was added with 200 µL of the eluate A (per 2 mL of puree). The samples were incubated for 24 h at 25 °C under shaking conditions. Following incubation, the purees samples were centrifuged at 13,000× *g* for 5 min in order to recover microbial cells for DNA extraction. DNA was extracted from the cell pellets with the power food microbial extraction kit (Qiagen, Madrid, Spain). The concentration of the extracted DNA was determined using Qubit 3.0 (Invitrogen, Waltham, MA, USA).

2.5. DNA Sequencing and Analysis

Once it was verified that the extracted samples met the quality conditions and the established minimum DNA concentration of 5 ng/L, the massive sequencing and bioinformatic analysis were entrusted to an external service (Fisabio, Valencia, Spain). Briefly, library preparation, quality assessment and sequence joining, bioinformatic analysis and metataxonomic analysis were performed as described in previous work [30]. The 16S rDNA gene V3–V4 region was amplified with the primers 5′TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG (F) and 5′GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC (R) using genomic DNA (5 ng/µL in 10 mM Tris pH 8.5) as a template. A Nextera XT Index Kit (Illumina, San Diego, CA, USA) was used for multiplexing, and the amplicon size (~550 bp) was verified on a Bioanalyzer DNA 1000 chip. After quality assessment with prinseq-lite program [31], the sequences were analyzed with the qiime2 pipeline [32]. The DADA2 pipeline [33] was used for denoising, the joining of paired ends and the depletion of chimera. The libraries were sequenced using a 2 × 300 bp paired-end run (MiSeq Reagent kit v3 (MS-

102-3001)) on a MiSeq Sequencer, according to the manufacturer's instructions (Illumina). Taxonomic affiliations were assigned using the Naive Bayesian classifier integrated in the quiime2 plugins, with Silva138_V3V4K as the database for taxonomic assignation.

2.6. Statistics

The statistical significance ($p < 0.05$) of the data corresponding to the culture-dependent microbiological analysis was determined using one-way ANOVA and Tukey's test. The alpha diversity richness estimators for species richness (Chao1) and community diversity (Shannon, Simpson) were obtained with the qiime2 pipeline. The average data of the three sample replicates was used for the statistical analyses of beta diversity. Data on the bacterial diversity were compared using principal coordinate analysis (PCoA) and comparing the different samples with Student's *t* test. For the different statistical analyses, the Past program (version 4.0) and R studio (version 4.2.2) were used. Krona representation was generated, starting from taxonomy proportion tables using the Krona hierarchical browser [34].

3. Results

3.1. Antimicrobial Activity of Eluates

The eluate purified with NaCl (E) produced inhibition zones in the range of 12 to 14 mm, depending on the test strain (Table 1). The eluate purified with acetonitrile (A) showed the highest activity against *S. aureus* CECT 976 and *B. cereus* LWL1. *Enterococcus faecalis* and *Listeria innocua* were not inhibited by any of the eluates. The eluents E and A showed no antibacterial activities.

Table 1. Antimicrobial activities of eluates obtained by cation exchange chromatography (E) and reversed-phase chromatography (A). Values indicate growth inhibition zone (in mm).

	Test Strains					
Eluate	***S. aureus* CECT 976**	***E. faecalis* S-47**	***S. enterica* CECT 3197**	***E. coli* E19**	***L. innocua* CECT 910**	***B. cereus* LWL1**
NaCl (E)	13	0	13	14	0	12
Acn (A)	16	0	9	9	0	17

3.2. Microbiological Counts

The microbiological counts in the different culture media and at different temperatures are shown in Table 2. It was observed that the total number of total aerobic mesophiles increased significantly ($p < 0.05$) with time, more at 25 °C than at 4 °C, both in the controls and in all the treated samples. For presumptive *Enterobacteriaceae* at 25 °C, the highest reductions of viable cell counts were obtained for treatment A, reducing the number of colonies below the detection limit on day 7. Significant differences were also observed in treatment E compared to untreated controls because the viable cell concentrations of presumptive *Enterobacteriaceae* did not increase during incubation, but rather remained at values close to time 0. Treatment P (inoculation with the *Paenibacillus* strain) also seemed to delay the proliferation of presumptive *Enterobacteriaceae* compared to controls at the end of storage. At 4 °C, presumptive *Enterobacteriaceae* were significantly lower at day 7 for all treatments, but the reduction of viable counts by treatment A was much lower compared to 25 °C. In the *Bacillus cereus* agar medium, significantly lower counts ($p < 0.05$) were observed mainly in treatments E and A at 25 °C with respect to the control. At 4 °C, viable cell counts decreased during incubation for all the samples, with no significant differences between controls and treated samples. The counts obtained on MRS agar (presumptive lactic acid bacteria) increased with time more at 25 °C than at 4 °C. Only treatments E (days 1 and 2) and P (day 2) showed significant differences with controls, at 25 °C.

Table 2. Viable cell counts of puree samples during incubation at 4 °C or 25 °C.

	T0	T1	T2	T7
Enterobacteriaceae 25 °C				
C	5.37 ± 0.02	4.70 ± 0.004	4.48 [b] ± 0.00	7.48 [a] ± 0.00
P	5.29 ± 0.03	5.84 [a] ± 0.03	4.48 [b] ± 0.00	6.59 [a,c] ± 0.01
E	4.11 [c] ± 0.01	4.95 [a] ± 0.05	4.48 [a] ± 0.00	4.48 [a,c] ± 0.00
A	5.25 ± 0.04	1.00 [b,c] ± 0.00	1.15 [b,c] ± 0.15	<1.00
Bacillus cereus agar 25 °C				
C	3.60 ± 0.03	5.31 [a] ± 0.01	6.65 [a] ± 0.02	4.48 [a] ± 0.00
P	5.05 ± 0.01	5.27 ± 0.01	5.05 [c] ± 0.03	4.48 [b] ± 0.00
E	3.61 ± 0.01	4.38 [a,c] ± 0.02	5.15 [a,c] ± 0.06	2.63 [b,c] ± 0.15
A	3.61 ± 0.03	5.35 [a] ± 0.12	5.31 [a,c] ± 0.05	3.09 [b,c] ± 0.05
Lactic acid bacteria 25 °C				
C	4.25 ± 0.03	7.48 [a] ± 0.00	7.94 [a] ± 0.08	8.72 [a] ± 0.02
P	4.37 ± 0.05	7.48 [a] ± 0.00	7.37 [a,c] ± 0.01	8.42 [a] ± 0.12
E	4.09 ± 0.01	6.48 [a,c] ± 0.00	7.36 [a,c] ± 0.00	8.57 [a] ± 0.09
A	4.25 ± 0.01	7.48 [a] ± 0.00	8.17 [a] ± 0.02	8.44 [a] ± 0.04
Total aerobic mesophiles 25 °C				
C	5.61 ± 0.01	7.48 [a] ± 0.00	8.06 [a] ± 0.00	8.64 [a] ± 0.05
P	5.84 ± 0.08	7.48 [a] ± 0.00	8.00 [a] ± 0.05	8.37 [a] ± 0.06
E	4.96 ± 0.00	6.48 [ac] ± 0.00	7.82 [a] ± 0.03	8.46 [a] ± 0.06
A	5.33 ± 0.03	7.48 [a] ± 0.00	8.01 [a] ± 0.05	8.35 [a] ± 0.04
Enterobacteriaceae 4 °C				
C	5.37 ± 0.02	5.05 [b] ± 0.05	5.49 ± 0.01	6.85 [a] ± 0.03
P	5.29 ± 0.03	4.76 [b] ± 0.02	5.53 ± 0.04	5.57 [c] ± 0.08
E	4.11 [c] ± 0.01	4.39 [c] ± 0.01	5.30 [a] ± 0.04	6.03 [a,c] ± 0.01
A	5.25 ± 0.04	4.7 [b] ± 0.14	5.61 ± 0.04	4.45 [b,c] ± 0.04
Bacillus cereus agar 4 °C				
C	3.60 ± 0.03	3.17 [b] ± 0.06	3.78 ± 0.04	2.26 [b] ± 0.08
P	5.05 ± 0.01	3.95 [b] ± 0.05	3.71 [b] ± 0.03	2.27 [b] ± 0.04
E	3.61 ± 0.01	3.26 [b] ± 0.06	3.61 ± 0.04	2.41 [b] ± 0.08
A	3.61 ± 0.03	3.17 [b] ± 0.13	3.58 ± 0.05	2.85 [b] ± 0.04
Lactic acid bacteria 4 °C				
C	4.25 ± 0.03	5.36 [a] ± 0.06	6.05 [a] ± 0.01	7.36 [a] ± 0.005
P	4.37 ± 0.05	5.16 [a] ± 0.03	5.87 [a] ± 0.01	7.29 [a] ± 0.02
E	4.09 ± 0.01	5.14 [a] ± 0.04	5.66 [a] ± 0.02	7.43 [a] ± 0.01
A	4.25 ± 0.00	5.08 [a] ± 0.02	5.61 [a] ± 0.03	7.39 [a] ± 0.02
Total aerobic mesophiles 4 °C				
C	5.61 ± 0.01	5.39 ± 0.03	6.17 [a] ± 0.03	7.64 [a] ± 0.10
P	5.84 ± 0.08	6.10 [a] ± 0.04	6.44 [a] ± 0.03	7.36 [a] ± 0.02
E	4.96 [c] ± 0.00	5.26 ± 0.02	6.02 [a] ± 0.07	7.32 [a] ± 0.08
A	5.33 ± 0.03	5.10 ± 0.03	5.99 [a] ± 0.03	8.02 [a] ± 0.02

Note: T, incubation time (days). Statistical significance ($p < 0.05$): [a], significantly higher than counts at time 0; [b], significantly lower than counts at time 0; [c], significantly lower than the corresponding control at the same incubation time.

3.3. Bacterial Biodiversity

The numbers of assigned reads and alpha diversity indices of controls and treated samples are shown in Table 3. The indices show that there was not a great diversity of species and there was dominance of a low number of species.

Table 3. Number of readings and alpha diversity indices at the genus level.

Sample	Nº Reads	Chao1	Shannon	Simpson
C1	123,226	25	1.703	0.749
C2	119,676	30	1.849	0.777
C3	127,885	29	1.724	0.739
P1	130,156	25	1.532	0.702
P2	112,884	28	1.885	0.787
P3	130,114	28	2.050	0.822
E1	124,532	28	1.224	0.494
E2	113,295	27	1.422	0.626
E3	119,261	26	1.814	0.787
A1	125,674	26	2.056	0.822
A2	121,055	29	1.988	0.812
A3	122,216	33	2.029	0.835

Note: C is control, P is the sample with *Paenibacillus*, E sample with NaCl eluate and A sample with Acetonitrile eluate. Chao 1 is an abundance-based estimator of species richness. The Shannon and Simpson indices are estimators of both species richness and species uniformity, with more emphasis on either richness (Shannon) or uniformity (Simpson).

The different operational taxonomic units (OTU) found in the puree samples were grouped into 4 phyla (Figure 1), of which *Pseudomonadota* were by far the main representatives, followed by *Bacillota*. *Actinobacteriota* and *Bacteroidota* had relative abundances that were below 0.5% in all samples. Remarkably, samples corresponding to treatment A showed a lower relative abundance of *Pseudomonadota* and a higher relative abundance of *Bacillota*.

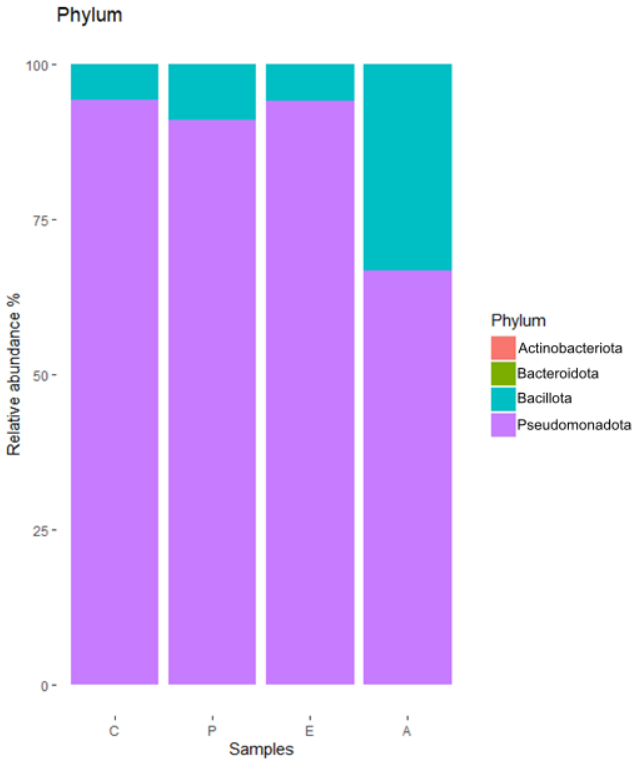

Figure 1. Bacterial diversity of puree samples at phylum level. C, untreated controls. P, puree inoculated with *Paenibacillus dendritiformis* UJA2219. E, puree treated with partially-purified extract

(1.5 M eluate). A, puree treated with partially-purified extract (60% acetonitrile). After 24 h incubation, total bacterial DNA was extracted from the purees and the 16S rDNA gene V3–V4 region was amplified and sequenced using Illumina technology. The DNA sequence reads obtained were assigned to operational taxonomic units (OTUs). OTUs were grouped by taxonomic level. The mean values of sample replicates are shown.

These phyla included representatives of 26 families (Figure 2). Of them, 8 families had relative abundances of at least 2% in at least one of the samples (*Pseudomonadaceae*, *Enterobacteriacea*, *Moraxellaceae*, *Bacillaceae*, *Enterococcaceae*, *Streptococcaceae*, *Xanthomonadaceae* and *Leuconostocaceae*). *Pseudomonadaceae* and *Enterobacteriace* were the families with higher relative abundances. The relative abundance of *Pseudomonadaceae* was highest in sample E and lowest in sample A. Family *Moraxellaceae* had the lowest relative abundance in sample E. *Xanthomonadaceae* only had a relative abundance > 2.0% in sample E. The following families belonging to the phylum *Bacillota* had the highest relative abundances in sample A and either low or very low relative abundances in the other samples: *Bacillaceae*, *Enterococcaceae* and *Streptococcaceae*.

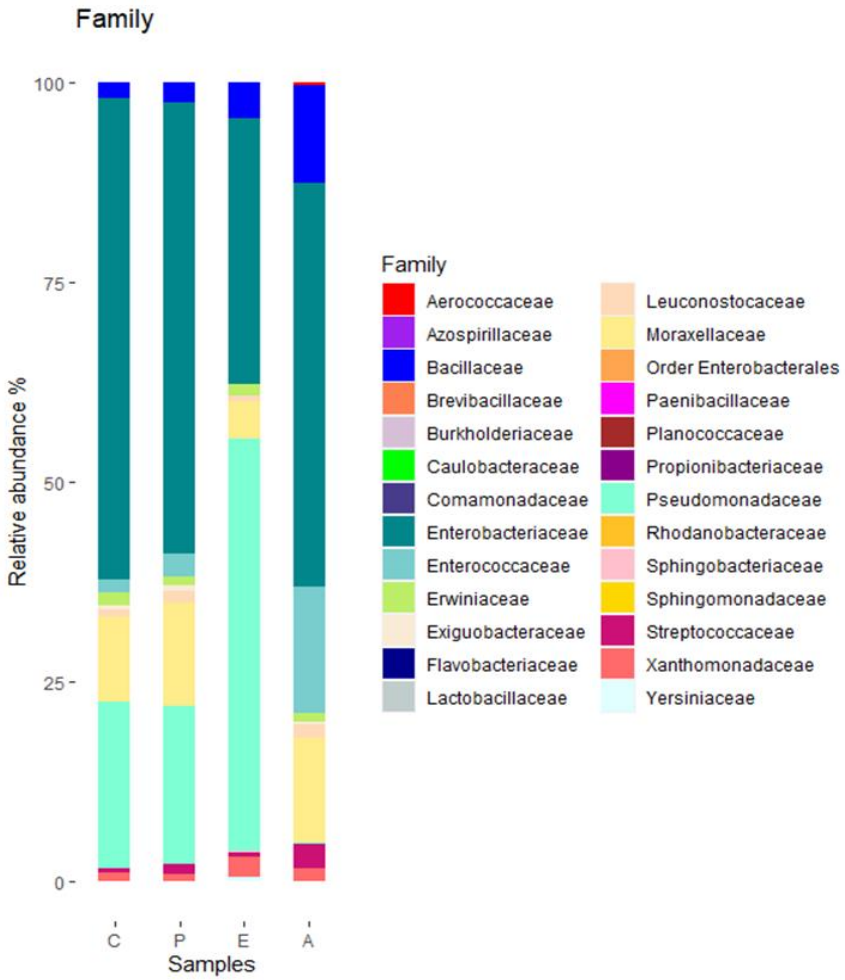

Figure 2. Bacterial diversity of puree samples at family level. C, untreated controls. P, puree inoculated with *Paenibacillus dendritiformis* UJA2219. E, puree treated with partially-purified extract (1.5 M eluate). A, puree treated with partially-purified extract (60% acetonitrile). After 24 h incubation, total bacterial DNA was extracted from the purees and the 16S rDNA gene V3–V4 region was amplified and sequenced using Illumina technology. The DNA sequence reads obtained were assigned to operational taxonomic units (OTUs). OTUs were grouped by taxonomic level. The mean values of sample replicates are shown.

At the genus level, a total of 37 genera were detected (Figure 3). Of them, 9 had relative abundances $\geq$ 2% in at least one sample (*Pseudomonas*, *Acinetobacter*, *Enterobacter*, *Klebsiella*, *Stenotrophomonas*, *Bacillus*, *Enterococcus*, *Lactococcus* and *Leuconostoc*). Most of the OTUs corresponding to family *Enterobacteriaceae* could not be assigned at the genus level. Among the assigned OTUs, genus *Enterobacter* was the main representative, followed by *Klebsiella*. *Escherichia-Shigella* had very low relative abundances. *Pseudomonadaceae* was represented mainly by genus *Pseudomonas*.

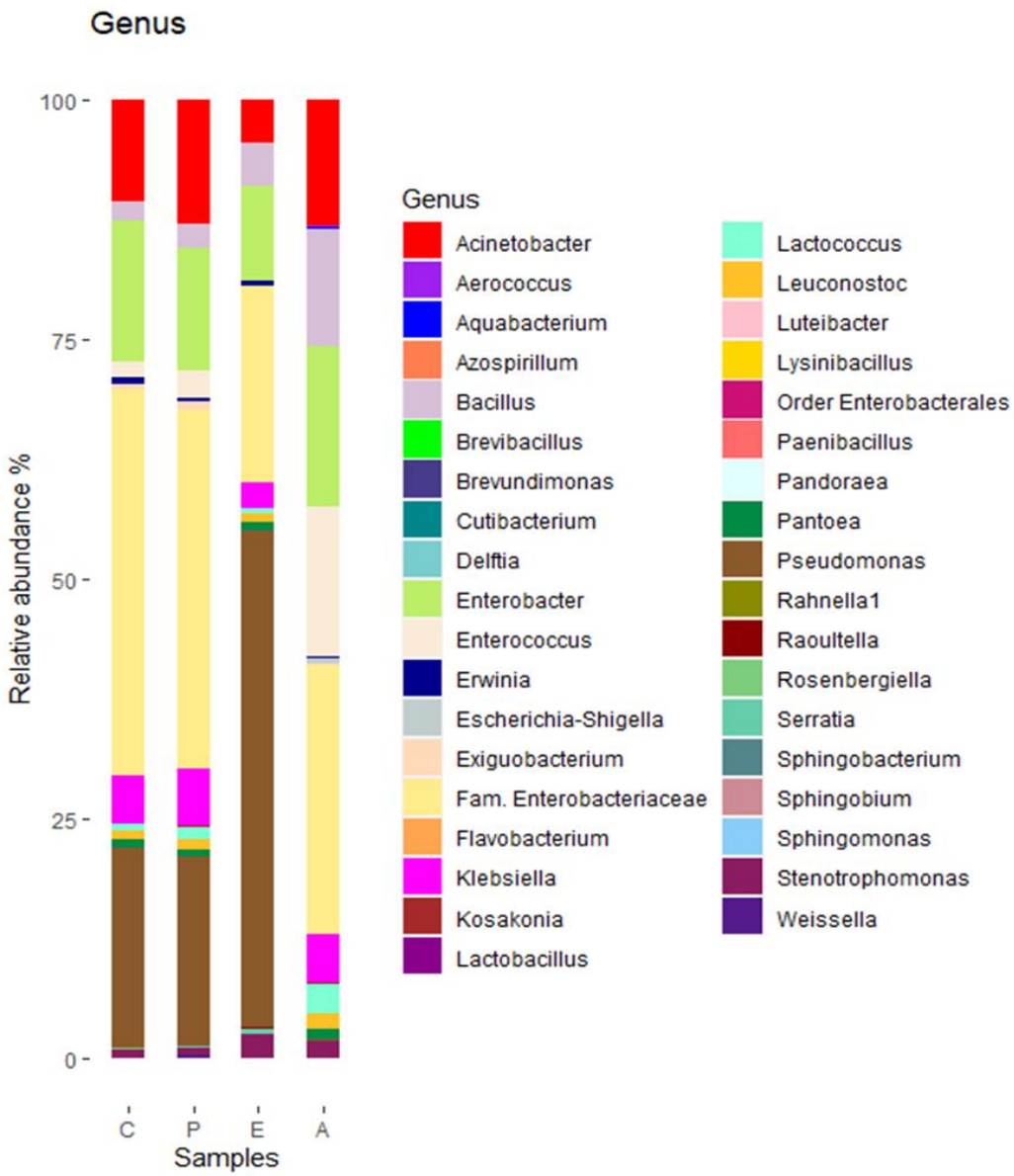

Figure 3. Bacterial diversity of puree samples at genus level. C, untreated controls. P, puree inoculated with *Paenibacillus dendritiformis* UJA2219. E, puree treated with partially-purified extract (1.5 M eluate). A, puree treated with partially-purified extract (60% acetonitrile). After 24 h incubation, total bacterial DNA was extracted from the purees and the 16S rDNA gene V3–V4 region was amplified and sequenced using Illumina technology. The DNA sequence reads obtained were assigned to operational taxonomic units (OTUs). OTUs were grouped by taxonomic level. The mean values of sample replicates are shown.

A global representation of the results of bacterial diversity showing the main taxonomic levels of the controls and treated samples is shown in Figure 4.

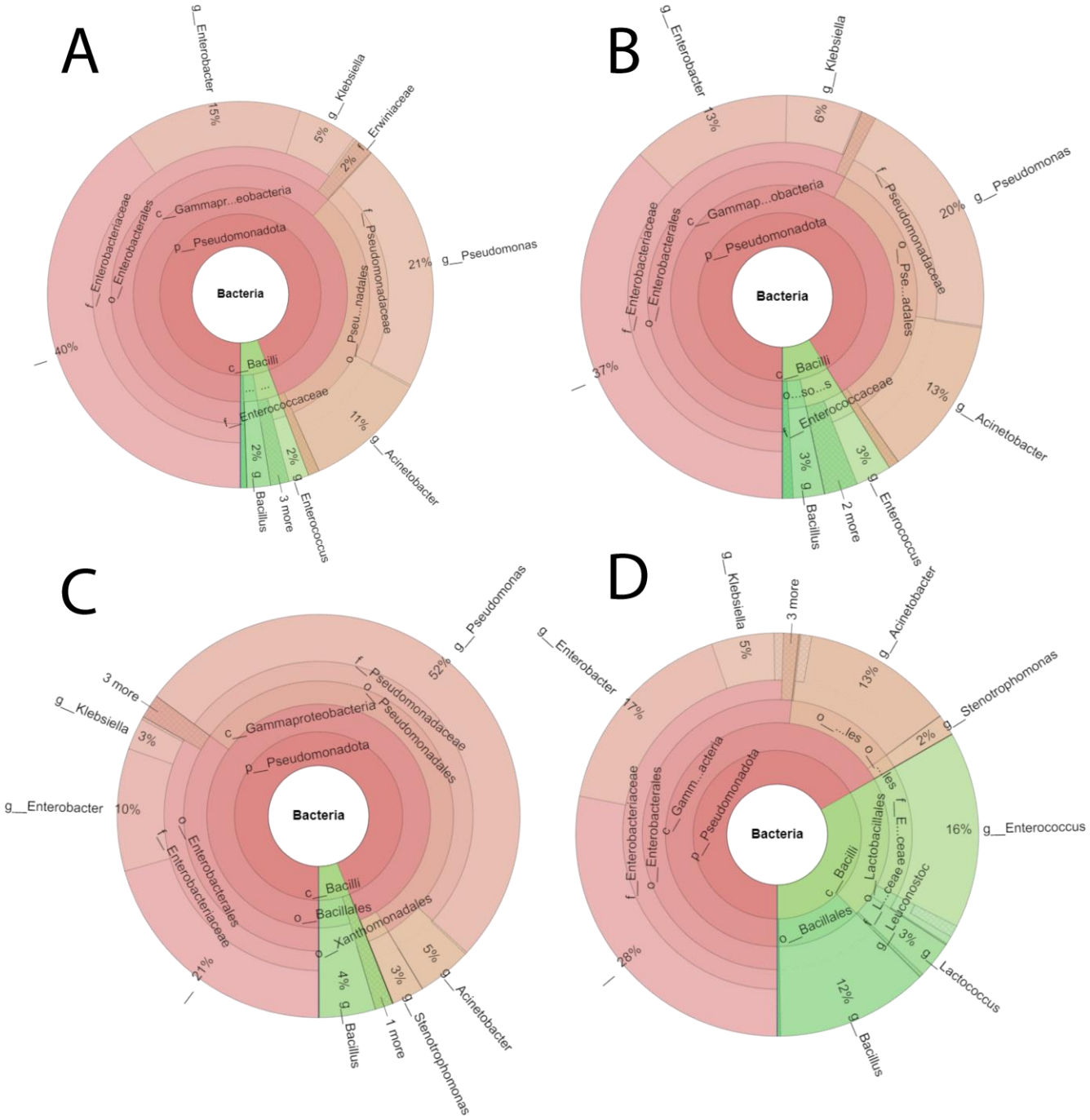

Figure 4. Global comparison of the means of biodiversity percentages obtained for the main taxonomic levels found in the puree samples, represented with Krona tools. (**A**) Untreated controls. (**B**) Puree inoculated with *Paenibacillus dendritiformis* UJA2219. (**C**) Puree treated with partially-purified extract (1.5 M eluate). (**D**) Puree treated with partially-purified extract (60% acetonitrile).

The statistical analysis of the differences in the main bacterial groups found in the puree samples with or without treatments is shown in Figure 5. *Enterobacteriaceae* (others) had relative abundances of 40% in both the control sample and in the sample inoculated with *Paenibacillus*; however, this percentage decreased significantly ($p < 0.05$) to 20% when treated with eluate E and 30% when treated with eluate A. Genus *Pseudomonas* was represented with quite similar percentages of approximately 20%, both in the control and in the sample inoculated with *Paenibacillus*, but instead it increased up to 50% in the sample treated with

eluate E and decreased significantly ($p < 0.05$), almost to zero, in the sample treated with eluate A.

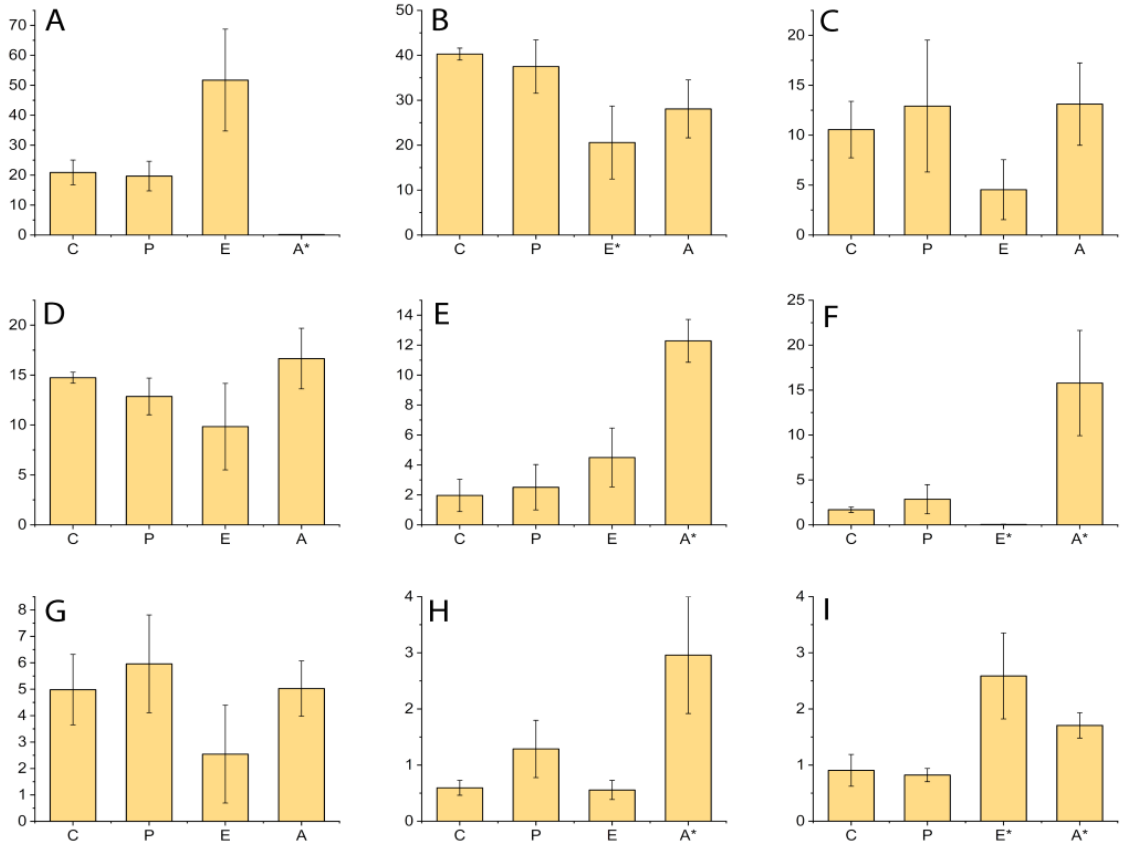

Figure 5. Comparison of the differences in relative abundance of the most representative genera found in the puree samples. Y axis represents relative abundance (average of three replicates ± standard deviation). X axis indicates sample type (C, controls; P, treatment with *Paenibacillus* strain; E, treatment with NaCl eluate; A, treatment with acetonitrile eluate. Asterisk denotes statistically significant ($p < 0.05$) differences with controls. Each graphic represents a different genus or taxonomic group: (**A**) *Pseudomonas*; (**B**) fam. *Enterobacteriaceae* (others); (**C**) *Acinetobacter*; (**D**) *Enterobacter*; (**E**) *Bacillus*; (**F**) *Enterococcus*; (**G**) *Klebsiella*; (**H**) *Lactococcus*; (**I**) *Stenotrophomonas*.

For the genera *Acinetobacter* and *Enterobacter*, which represented between 10% and 14% of the total OTUs, treatment with the NaCl eluate reduced their relative abundances by at least half. The relative abundance of *Acinetobacter* increased after *Paenibacillus* inoculation or treatment with acetonitrile eluate. The treatment with the acetonitrile eluate (A) significantly ($p < 0.05$) increased the relative abundance of *Bacillus*, *Enterococcus* and *Lactococcus*. Treatment with NaCl eluate significantly ($p < 0.05$) decreased the population of *Enterococcus* while it significantly ($p < 0.05$) increased that of *Stenotrophomonas*.

The differences between samples were also analyzed comparing the global results of relative abundances at the genus level using principal coordinate analysis (PCoA). The results (Figure 6) revealed that the control sample and the sample inoculated with *Paenibacillus* are very similar; however, there is a large difference between the samples treated with the eluates with respect to the control and to each other.

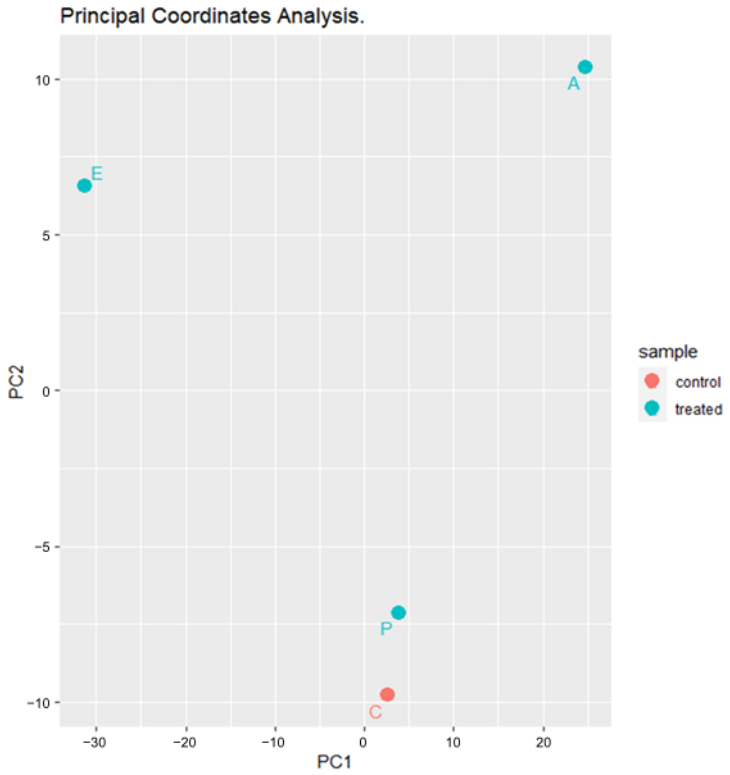

Figure 6. Principal coordinates analysis of puree samples treated with elutes or with *Paenibacillus dendritiformis* UJA2219. C, untreated controls. P, puree inoculated with *P. dendritiformis* UJA2219. E, puree treated with partially-purified extract (1.5 M eluate). A, puree treated with partially-purified extract (60% acetonitrile).

4. Discussion

The results obtained in the present study indicate that *P. dendritiformis* UJA2219 produces antimicrobial activity against bacteria at risk in foods as *E. coli* E19, *S. enterica* CECT 3197, *S. aureus* CECT 976 and *B. cereus* LWL1. The antibacterial activity can be recovered from cultured broths by cation exchange chromatography and also by reversed-phase chromatography.

To study the antimicrobial effect of these partially purified fractions, a food model was chosen, which was a homemade vegetable puree. Previous studies carried out in our group [6] indicated that the *Paenibacillus* genus presented antimicrobial activity and modified the growth of pathogens present in food. The annotation of the genome of *P. dendritiformis* UJA2219 (Mena et al., in preparation) suggests that this bacterial strain may produce secondary metabolites identified as 1 RiPP protein (paeninodin) and NRP proteins, such as paenibacterin, pelgipectin and paenilamicin. It could be that the addition of *P. dendritiformis* or any of its metabolites to a food model, in this case a homemade vegetable puree, generates an antimicrobial effect and/or a modification of microbial biodiversity.

The results obtained on microbial counts in puree inoculated with *P. dendritiformis* UJA2219 indicated that the bacterium failed to inhibit the puree microbiota, except for the weak decrease of presumptive *Enterobacteriaceae* detected at day 7 of incubation at 25 °C. These results could be due to a low production of antimicrobials in the puree by the inoculated strain. In situ production of antimicrobial substances is markedly influenced by many factors, such as pH, aeration, incubation temperature, time of incubation, available nutrients, the food matrix and the competing microbiota [35]. In published studies of the efficacy of other *Paenibacillus* strains in food, it has been shown to reduce the populations of *Bacillus subtilis* and *Listeria* in milk, which makes it promising for the biopreservation of dairy products [36]. However, strain UJA2219 did not show antilisteria activity in the

preliminary tests. Another *Paenibacillus* strain was effective against *Clostridium botulinum* in puree [37]. Most of these studies are based on assays performed with the strain in question, not with the purified fractions.

In the present study, the treatments carried out indicated that partially-purified preparations from *P. dendritiformis* UJA2219 cultured broths exerted an antimicrobial effect on the vegetable puree incubated at 4 °C and 25 °C that depended on the bacterial group. Antibacterial activity was more pronounced at 25 °C than under refrigerated conditions, suggesting that it could help the preservation of these purees outside of refrigeration. At 25 °C, for all mesophilic aerobic bacteria and lactic acid bacteria, an efficacy of the eluate from NaCl was observed during the first two days, but not after 7 days. For presumptive *Bacillus*, an efficacy of the two eluates (A and E) was observed after 7 days. However, from the inhibitory spectrum of the eluates, we would expect a greater reduction of the *Bacillus* population. Nevertheless, we should consider that there may be great differences in strain sensitivity, and that the possible *Bacillus* strains found in the puree may not necessarily be as sensitive as the LWL1 strain used in the assays of antibacterial activity. While eluate E seemed to inhibit the proliferation of presumptive *Enterobacteriaceae* in the samples incubated at 25 °C, the eluate A showed a remarkable bactericidal effect from day 1 of incubation, decreasing the CFU/mL levels below the detection limits after 7 days. On the other hand, at 4 °C, the effect on presumptive *Enterobacteriaceae* (as well as the other bacterial groups) was markedly reduced. The lower antibacterial effects observed for the partially purified preparations in the assays carried out at 4 °C could be explained by a lower diffusion of the antimicrobials in the food matrix. Another possible explanation could be that the antimicrobial(s) only act on actively growing cells. Previous studies have shown that the activity of some bacteriocins may be energy-dependent, as in the case of nisin [38] or microcin L [39]. In another example, the killing activity of enterocin AS-48 against *Staphylococcus aureus* CECT 976 in culture medium decreased markedly for the incubation temperatures of 10 °C or 4 °C compared to 37 °C [40].

Regarding the impact of *Paenibacillus* and its partially-purified extracts on bacterial diversity, the results obtained in the present study indicate that inoculation with the UJA2219 strain had no remarkable impact on the microbiota of the puree. These results agree with those obtained in the culture-dependent experiments in which the inoculated strain failed to inhibit proliferation of the different bacterial groups analyzed. Regarding treatments with culture extracts, the observed effect for the most representative genera of the samples (*Pseudomonas* and *Enterobacteriaceae*) was different depending on the treatments: in the case of the genus *Pseudomonas* the eluate with NaCl (E) makes the population increase in relative abundance almost twice while the treatment with the eluate of acetonitrile (A) decreases it to undetectable limits. In the case of *Enterobacteriaceae*, it was the treatment with the NaCl eluate (E) that had the greatest effect, reducing the relative abundance of this group by half, while the eluate from the purification with acetonitrile (A) did not have such a marked effect on this population. These results seem to be contradictory with those obtained by the culture-dependent approach (according to which eluate A had a remarkable effect on the population of presumptive *Enterobacteriaceae*). However, it should be taken into account that the selective medium used (EMB) also supports the growth of *Pseudomonas* [41] and, while *Pseudomonas* are non-fermentative, they may be difficult to differentiate from the true fermentative *Enterobacteriaceae* in mixed growth. Therefore, the results could be explained assuming that the counts obtained on EMB agar as presumptive *Enterobacteriaceae* include, at least in part, *Pseudomonas*.

An additional effect of treatment with eluate A was an increase in the relative abundance of *Enterococcus*. While neither eluate A or B had antibacterial activity on the test strain *E. faecalis* S-47, the increase in the relative abundance of *Enterococcus* in samples treated with eluate A could be linked to the observed decrease in the relative abundance of *Pseudomonas*. It could be speculated that the *Pseudomonas* population acts as a competitor against enterococci. In addition to competition for nutrients, *Pseudomonas* may produce antibacterial peptides, such as pyocins, lectin-like bacteriocins and modified microcins [42]. These results

illustrate the complex effects of incorporating antimicrobial substances into food systems and how the culture-independent methods such as next-generation sequencing provide new insights on the complexity of microbial interactions in foods [43].

Furthermore, the observed differences in the effects of partially purified culture extracts on bacterial diversity could also be interpreted considering that *Paenibacillus* UJA2219 may produce different antimicrobial substances. Thus, it is possible that the antimicrobial peptides recovered in the acetonitrile eluate are different from those recovered by cation exchange chromatography. *Paenibacillus* may produce a mixture of lasso peptides, lipopeptides and bacteriocins [22,24]. Many of these peptides are cationic and could be recovered from culture supernatants by cation exchange [24]. Others, however, may be amphipathic or hydrophobic (as in the case of lipopeptides) and show a low solubility in the water used as eluent for cation exchange. Instead, hydrophobic peptides would have a higher solubility in acetonitrile. Therefore, it is tempting to suggest that the different eluates are enriched in different peptides that act differently on each microbial population and therefore have different effects on the bacterial diversity. Furthermore, the eluent itself may also affect the peptide solubility and final activity. Furthermore, the results were expressed in terms of relative abundance and therefore the observed increase in relative abundance for a given population may simply be the result of a decrease in the relative abundance of the other populations in the sample.

At present, it is important for the food industry not only to evaluate the relationships between the different microbial populations present in a food, but also to know their behavior when they are subjected to treatments with secondary metabolites with antimicrobial potential, as is the case of *P. dendritiformis* UJA2219. Further experiments with the purified peptides are needed in order to better understand their impact on the bacterial communities of food systems and their potential as new biopreservatives.

5. Conclusions and Future Work

Results from the present study confirm that the partially-purified cultured broths from *P. dendritiformis* UJA2219 show antibacterial activity in a model food system and induce selective changes in the food microbiota. This opens the way for future studies on the effects of preparations derived from strain UJA2219 in food preservation, either by direct application in other food systems or for the development of active coatings aimed at controlling food spoilage or pathogenic bacteria. Strain UJA2219 was selected because it produces antimicrobial substances against four relevant foodborne pathogens (*E. coli*, *S. enterica*, *S. aureus* and *B. cereus*). The results obtained by culture-dependent methods could be broadened by doing challenge tests with strains or cocktails of strains belonging to these bacterial species, in order to determine the best conditions for application of the antibacterial extracts. One limitation of the present study is the use of partially-purified extracts (which may contain more than one antimicrobial substance, as suggested also by the preliminary data on the genome of the bacterium). The purification of the inhibitory substance(s) produced by strain UJA2219 is needed in order to evaluate its potential for application in food preservation and to establish the inhibitory spectrum and stability in food systems of the inhibitory substance(s) produced. Further studies are also needed to combine culture-dependent and culture-independent approaches in order to obtain more information on the effects of said antimicrobials on the dynamics of the bacterial populations of the food system and the influence of factors such as storage conditions and time.

Author Contributions: Conceptualization, M.J.G.; methodology, M.J.G., L.M.; formal analysis, L.M., M.J.G. and A.G.; investigation, L.M. and M.J.G.; writing—review and editing, M.J.G., L.M. and A.G.; supervision, M.J.G.; project administration, M.J.G.; funding acquisition, M.J.G. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Ministerio de Ciencia e Innovación (Unión Europea, Agencia Estatal de Investigación, Programa Ramón y Cajal), grant number RYC-2017-23077. L.M. received a research grant from University of Jaén (Ayudas Predoctorales para la Formación de Personal

Investigador del Plan de Apoyo a la Investigación de la Universidad de Jaén 2019-2020), grant number PIPFP91. The APC was funded by University of Jaén (AGR230).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author. The data are not publicly available due to privacy.

Acknowledgments: This work was supported by the Ramón y Cajal Program, action co-financed by Ministry of Economy and Competitiveness (MINECO) and European Union and pre-doctoral grants for the training of research personnel under Action 9a of the Operational Plan for Research Support in the University of Jaén.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

References

1. Fernández-López, J.; Botella-Martínez, C.; Navarro-Rodríguez, C.; Vera, D.; Viuda-Martos, M.; Sánchez-Zapatas, E.; Pérez-Álvarez, J.A. Vegetable Soups and Creams: Raw Materials, Processing, Health Benefits, and Innovation Trends. *Plants* **2020**, *9*, 1769. [CrossRef] [PubMed]
2. Liu, R.H. Health-Promoting Components of Fruits and Vegetables in the Diet. *Adv. Nutr.* **2013**, *4*, 384S–392S. [CrossRef]
3. Kowalska, B.; Szczech, M. Differences in Microbiological Quality of Leafy Green Vegetables. *Ann. Agric. Environ. Med.* **2022**, *29*, 238–245. [CrossRef] [PubMed]
4. Carlin, F.; Guinebretiere, M.H.; Choma, C.; Pasqualini, R.; Braconnier, A.; Nguyen-The, C. Spore-Forming Bacteria in Commercial Cooked, Pasteurised and Chilled Vegetable Purees. *Food Microbiol.* **2000**, *17*, 153–165. [CrossRef]
5. Schoeni, J.L.; Lee Wong, A.C. *Bacillus cereus* Food Poisoning and Its Toxins. *J. Food Prot.* **2005**, *68*, 636–648. [CrossRef] [PubMed]
6. Grande, M.J.; Abriouel, H.; López, R.L.; Valdivia, E.; Omar, N.B.; Martínez-Cañamero, M.; Gálvez, A. Efficacy of Enterocin AS-48 against Bacilli in Ready-to-Eat Vegetable Soups and Purees. *J. Food Prot.* **2007**, *70*, 2339–2345. [CrossRef]
7. Pedersen, P.B.; Bjørnvad, M.E.; Rasmussen, M.D.; Petersen, J.N. Cytotoxic Potential of Industrial Strains of *Bacillus* sp. *Regul. Toxicol. Pharmacol.* **2002**, *36*, 155–161. [CrossRef] [PubMed]
8. Kumariya, R.; Garsa, A.K.; Rajput, Y.S.; Sood, S.K.; Akhtar, N.; Patel, S. Bacteriocins: Classification, Synthesis, Mechanism of Action and Resistance Development in Food Spoilage Causing Bacteria. *Microb. Pathog.* **2019**, *128*, 171–177. [CrossRef] [PubMed]
9. Gálvez, A.; Grande Burgos, M.J.; Lucas Lopez, R.; Pérez Pulido, R. *Food Biopreservation*; Hartel, R.W., Ed.; Springer: New York, NY, USA, 2014; ISBN 9781493920280.
10. Choyam, S.; Srivastava, A.K.; Shin, J.H.; Kammara, R. Ocins for Food Safety. *Front. Microbiol.* **2019**, *10*, 1736. [CrossRef]
11. Lahiri, D.; Nag, M.; Dutta, B.; Sarkar, T.; Pati, S.; Basu, D.; Abdul Kari, Z.; Wei, L.S.; Smaoui, S.; Wen Goh, K.; et al. Bacteriocin: A Natural Approach for Food Safety and Food Security. *Front. Bioeng. Biotechnol.* **2022**, *10*, 1005918. [CrossRef]
12. Rendueles, C.; Duarte, A.C.; Escobedo, S.; Fernández, L.; Rodríguez, A.; García, P.; Martínez, B. Combined Use of Bacteriocins and Bacteriophages as Food Biopreservatives: A Review. *Int. J. Food Microbiol.* **2022**, *368*, 109611. [CrossRef] [PubMed]
13. Caulier, S.; Nannan, C.; Gillis, A.; Licciardi, F.; Bragard, C.; Mahillon, J. Overview of the Antimicrobial Compounds Produced by Members of the *Bacillus subtilis* Group. *Front. Microbiol.* **2019**, *10*, 302. [CrossRef] [PubMed]
14. Abriouel, H.; Franz, C.M.A.P.; Omar, N.B.; Galvez, A. Diversity and Applications of *Bacillus* Bacteriocins. *FEMS Microbiol. Rev.* **2011**, *35*, 201–232. [CrossRef] [PubMed]
15. Basi-Chipalu, S.; Sthapit, P.; Dhital, S. A review on characterization, applications and structure-activity relationships of *Bacillus* species-produced bacteriocins. *Drug Discov. Ther.* **2022**, *16*, 55–62. [CrossRef] [PubMed]
16. Mercado, V.; Olmos, J. Bacteriocin Production by *Bacillus* Species: Isolation, Characterization, and Application. *Probiotics Antimicrob. Proteins* **2022**, *14*, 1151–1169. [CrossRef] [PubMed]
17. Zhang, B.; Xu, L.; Ding, J.; Wang, M.; Ge, R.; Zhao, H.; Zhang, B.; Fan, J. Natural Antimicrobial Lipopeptides Secreted by *Bacillus* Spp. and Their Application in Food Preservation, a Critical Review. *Trends Food Sci. Technol.* **2022**, *127*, 26–37. [CrossRef]
18. Sirota-Madi, A.; Olender, T.; Helman, Y.; Finkelshtein, I.B.; Roth, D.; Hagai, E.; Leshkowitz, D.; Brodsky, L.; Galatenko, V.; Nikolaev, V.; et al. Genome Sequence of the Pattern-Forming Social Bacterium: *Paenibacillus dendritiformis* C454 Chiral Morphotype. *J. Bacteriol.* **2012**, *194*, 2127–2128. [CrossRef]
19. Be'er, A.; Ariel, G.; Kalisman, O.; Helman, Y.; Sirota-Madi, A.; Zhang, H.P.; Florin, E.L.; Payne, S.M.; Ben-Jacob, E.; Swinney, H.L. Lethal Protein Produced in Response to Competition between Sibling Bacterial Colonies. *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 6258–6263. [CrossRef]
20. Gonzalez-Pastor, J.E.; Hobbs, E.C.; Losick, R. Cannibalism by Sporulating Bacteria. *Science* **2003**, *301*, 510–513. [CrossRef]
21. Claverys, J.P.; Håvarstein, L.S. Cannibalism and Fratricide: Mechanisms and Raisons d'être. *Nat. Rev. Microbiol.* **2007**, *5*, 219–229. [CrossRef]

22. Grady, E.N.; MacDonald, J.; Liu, L.; Richman, A.; Yuan, Z.-C. Current knowledge and perspectives of *Paenibacillus*: A review. *Microb. Cell Fact.* **2016**, *15*, 203. [CrossRef] [PubMed]
23. Jangra, M.; Randhawa, H.K.; Kaur, M.; Srivastava, A.; Maurya, N.; Patil, P.P.; Jaswal, P.; Arora, A.; Patil, P.B.; Raje, M.; et al. Purification, Characterization and in Vitro Evaluation of Polymyxin a from *Paenibacillus dendritiformis*: An Underexplored Member of the Polymyxin Family. *Front. Microbiol.* **2018**, *9*, 2864. [CrossRef] [PubMed]
24. Zhu, S.; Hegemann, J.D.; Fage, C.D.; Zimmermann, M.; Xie, X.; Linne, U.; Marahiel, M.A. Insights into the Unique Phosphorylation of the Lasso Peptide Paeninodin. *J. Biol. Chem.* **2016**, *291*, 13662–13678. [CrossRef]
25. Kumar, K.; Verma, A.; Pal, G.; Anubha; White, J.F.; Verma, S.K. Seed Endophytic Bacteria of Pearl Millet (*Pennisetum glaucum* L.) Promote Seedling Development and Defend Against a Fungal Phytopathogen. *Front. Microbiol.* **2021**, *12*, 774293. [CrossRef]
26. Yadav, M.; Dubey, M.K.; Upadhyay, R.S. Systemic Resistance in Chilli Pepper against Anthracnose (Caused by *Colletotrichum truncatum*) Induced by *Trichoderma harzianum*, *Trichoderma asperellum* and *Paenibacillus dendritiformis*. *J. Fungi* **2021**, *7*, 307. [CrossRef] [PubMed]
27. Lapidot, D.; Dror, R.; Vered, E.; Mishli, O.; Levy, D.; Helman, Y. Disease protection and growth promotion of potatoes (*Solanum tuberosum* L.) by *Paenibacillus dendritiformis*. *Plant Pathol.* **2014**, *64*, 545–551. [CrossRef]
28. Abriouel, H.; Valdivia, E.; Martínez-Bueno, M.; Maqueda, M.; Gálvez, A. A Simple Method for Semi-Preparative-Scale Production and Recovery of Enterocin AS-48 Derived from *Enterococcus faecalis* subsp. *liquefaciens A-48-32*. *J. Microbiol. Methods* **2003**, *55*, 599–605. [CrossRef] [PubMed]
29. Tagg, J.R.; McGiven, A.R. Assay System for Bacteriocins. *Appl. Microbiol.* **1971**, *21*, 943. [CrossRef]
30. Rodriguez-López, J.; Grande, M.J.; Pérez-Pulido, R.; Galvez, A.; Lucas, R. Impact of High-Hydrostatic Pressure Treatments Applied Singly or in Combination with Moderate Heat on the Microbial Load, Antimicrobial Resistance, and Bacterial Diversity of Guacamole. *Microorganisms* **2020**, *8*, 909. [CrossRef]
31. Schmieder, R.; Edwards, R. Quality Control and Preprocessing of Metagenomic Datasets. *Bioinformatics* **2011**, *27*, 863–864. [CrossRef]
32. Caporaso, J.G.; Lauber, C.L.; Walters, W.A.; Berg-Lyons, D.; Lozupone, C.A.; Turnbaugh, P.J.; Fierer, N.; Knight, R. Global Patterns of 16S RRNA Diversity at a Depth of Millions of Sequences per Sample. *Proc. Natl. Acad. Sci. USA* **2011**, *108*, 4516–4522. [CrossRef] [PubMed]
33. Callahan, B.J.; McMurdie, P.J.; Rosen, M.J.; Han, A.W.; Johnson, A.J.A.; Holmes, S.P. DADA2: High-Resolution Sample Inference from Illumina Amplicon Data. *Nat. Methods* **2016**, *13*, 581–583. [CrossRef] [PubMed]
34. Ondov, B.D.; Bergman, N.H.; Phillippy, A.M. Interactive Metagenomic Visualization in a Web Browser. *BMC Bioinform.* **2011**, *12*, 385. [CrossRef] [PubMed]
35. Gálvez, A.; Abriouel, H.; López, R.L.; Omar, N. Ben Bacteriocin-Based Strategies for Food Biopreservation. *Int. J. Food Microbiol.* **2007**, *120*, 51–70. [CrossRef]
36. El-Sharoud, W.M.; Zalma, S.A.; Yousef, A.E. Inducing the Production of the Bacteriocin Paenibacillin by *Paenibacillus polymyxa* through Application of Environmental Stresses with Relevance to Milk Bio-Preservation. *Int. J. Food Microbiol.* **2022**, *371*, 109637. [CrossRef]
37. Girardin, H.; Albagnac, C.; Dargaignaratz, C.; Nguyen-The, C.; Carlin, F. Antimicrobial Activity of Foodborne *Paenibacillus* and *Bacillus* spp. against *Clostridium botulinum*. *J. Food Prot.* **2002**, *65*, 806–813. [CrossRef]
38. Bruno, M.E.C.; Montville, T.J. Common Mechanistic Action of Bacteriocins from Lactic Acid Bacteria. The Influence of Four Bacteriocins from Lactic Acid Bacteria on the Proton Motive Force (PMF) of Sensitive. *Appl. Environ. Microbiol.* **1993**, *59*, 3003–3010. [CrossRef]
39. Morin, N.; Lanneluc, I.; Connil, N.; Cottenceau, M.; Pons, A.M.; Sablé, S. Mechanism of Bactericidal Activity of Microcin L in *Escherichia coli* and *Salmonella enterica*. *Antimicrob. Agents Chemother.* **2011**, *55*, 997–1007. [CrossRef]
40. Ananou, S.; Valdivia, E.; Martínez Bueno, M.; Gálvez, A.; Maqueda, M. Effect of Combined Physico-Chemical Preservatives on Enterocin AS-48 Activity against the Enterotoxigenic *Staphylococcus aureus* CECT 976 Strain. *J. Appl. Microbiol.* **2004**, *97*, 48–56. [CrossRef]
41. Jeppesen, C. Media for *Aeromonas* spp., *Plesiomonas shigelloides* and *Pseudomonas* spp. from Food and Environment. *Int. J. Food Microbiol.* **1995**, *26*, 25–41. [CrossRef]
42. Ghequire, M.G.K.; De Mot, R. Ribosomally Encoded Antibacterial Proteins and Peptides from *Pseudomonas*. *FEMS Microbiol. Rev.* **2014**, *38*, 523–568. [CrossRef] [PubMed]
43. Ercolini, D. High-Throughput Sequencing and Metagenomics: Moving Forward in the Culture-Independent Analysis of Food Microbial Ecology. *Appl. Environ. Microbiol.* **2013**, *79*, 3148–3155. [CrossRef] [PubMed]