

## 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, 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 home-made 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. Best results were obtained at 25 °C with the RP extract, decreasing counts of 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 *Proteobacteria* (mainly Fam. *Pseudomonadaceae* -gen. *Pseudomonas*- and *Enterobacteriaceae* as most abundant groups). Greatest differences in bacterial diversity were obtained in the puree treated with RP extract, showing a decrease in the relative abundance of *Proteobacteria* (especially gen. *Pseudomonas*) and an increase of *Firmicutes* (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*

## 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 by 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].

*Paenibacillus dendritiformis*, is a Gram positive microorganism, which lives in the soil, forms spores and has the ability to change between different morphotypes [9]. 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 [10]. 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 [11]. 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 [12]. About the production and isolation of antimicrobial compounds from *P. dendritiformis*, it has been seen that it can produce antimicrobial substances, which were purified using various chromatographic techniques. These compounds had characteristics similar to polymyxins but with more efficacy than commercial polymyxins and at the same time much less toxicity, which makes them a good alternative for the search for new antimicrobial substances against gram-negative and multiresistant bacteria [13].

Therefore, antimicrobial substances from *Paenibacillus* have a 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 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 carries genetic determinants related to other putative antimicrobial peptides 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 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 also 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, Scharlau, Barcelona, Spain) at 30 °C with shaking was inoculated (1% wt/vol) in 2 l of complex medium CM [14] 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 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). The gels were then washed with 50 ml distilled water. Eluants (45 ml each) were 1.5 M NaCl in distilled water (for the cation Exchange gel), 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, Scharlau) according to Tagg & McGiven, 1971 [15]. The bacteria used for sensitivity tests were from our laboratory collection or from the Spanish Type Culture Collection (CECT, Burjassot, Valencia, Spain): *Escherichia coli* E19, *Staphylococcus aureus* CECT 976, *Salmonella enterica* CECT 3197 and *Bacillus cereus* LWL1.

### 2.2 Preparation of the puree

We prepare a homemade vegetable puree 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). 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 control (without any addition of antimicrobials). The second tube was inoculated (1% vol/vol) with an overnight culture of *Paenibacillus dendritiformis* strain UJA2219 in order to test the potential of the bacterium for direct antagonism in the puree. To the third tube we added 500 µl of the partially purified with NaCl eluate (E) and to the last sample we 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 y 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 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 control. The second set was inoculated (1%, vol/vol) with an overnight culture of *Paenibacillus dendritiformis* UJA2219 as described above. The third set was added 200 µl of the eluate E (per 2ml of puree) and the last set was added 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 x 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 extracted DNA was determined using Qubit 3.0 (Invitrogene, Massachusetts, 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 done as described in previous work [16]. The libraries were sequenced using a 2x300 bp paired-end run (MiSeq Reagent kit v3 (MS-102-3001)) on a MiSeq Sequencer according to manufacturer's instructions (Illumina). Taxonomic affiliations were assigned using the Naive Bayesian classifier integrated in quime2 plugins, with Silva138\_V3V4K as database for taxonomic assignation.

### 2.6. Statistics

The statistical significance of the data corresponding to the culture-dependent microbiological analysis was determined using one-way ANOVA and Tukey's test. Data on bacterial diversity were compared by Principal Coordinate Analysis (PCoA) and comparing the different samples by 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 tools [17] were also used to represent the different percentages of diversity.

## 3. Results

### 3.1. Antimicrobial activity of eluates

The eluate purified with NaCl (E) produced an inhibition zone of 13 mm diameter for *S. aureus* CECT 976, 13 mm for *S. enterica* CECT 3197, 14 mm for *E. coli* E19 and 12 mm for *B. cereus* LWL1, while inhibition caused by the eluate purified with acetonitrile (A) was 16 mm in diameter for *S.*

aureus CECT 976, 9 mm for *S. enterica* CECT 3197, 9 mm for *E. coli* E19 and 17 mm for *B. cereus* LWL1. The eluants E and A showed no antibacterial activities.

### 3.2. Microbiological counts

The microbiological counts in the different culture media and at different temperatures are shown in table 1. 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 of the treated samples. For presumptive Enterobacteriaceae at 25 °C, highest reductions of viable cell counts were obtained for treatment A, reducing even on day 7 the number of colonies below the detection limit. 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 proliferation of presumptive Enterobacteriaceae compared to controls at the end of storage. By contrast, in the samples incubated at 4 °C no significant differences were observed for presumptive Enterobacteriaceae. In the *Bacillus cereus* agar medium, significantly lower counts ( $p < 0.05$ ) were observed in treatments E and A at 25 °C with respect to the control, but not for treatment P. 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 ( $p < 0.05$ ), but no statistically significant differences were observed between controls and treated samples.

**Table 1.** Viable cell counts of puree samples during incubation at 4 °C or 25 °C.

	T0	T1	T2	T7
<i>Enterobacteriaceae</i>				
25 °C				
C	5.37±0.02 <sup>h</sup>	4.70±0.004 <sup>f g h i</sup>	4.48±0.00 <sup>f g h i</sup>	7.48±0.00 <sup>g h</sup>
P	5.29±0.03 <sup>h</sup>	5.84±0.03 <sup>a f g h</sup>	4.48±0.00 <sup>f g h i</sup>	6.59±0.007 <sup>a g h</sup>
E	4.11±0.01 <sup>a b d h</sup>	4.95±0.05 <sup>b g h i</sup>	4.48±0.00 <sup>f g h i</sup>	4.48±0.00 <sup>a b g h i</sup>
A	5.25±0.04	1.00±0 <sup>a b c f g h i</sup>	1.15±0.15 <sup>a b c f g h i</sup>	<1
<i>Bacillus cereus</i>				
Agar 25 °C				
C	3.60±0.03 <sup>e g h</sup>	5.31±0.01 <sup>g h</sup>	6.65±0.02 <sup>g h</sup>	4.48±0.00 <sup>e g h</sup>
P	5.05±0.01 <sup>a c d e h</sup>	5.27±0.007 <sup>g h</sup>	5.05±0.03 <sup>a g h</sup>	4.48±0.00 <sup>e g h</sup>
E	3.61±0.01 <sup>e g h</sup>	4.38±0.02 <sup>a b d e g h</sup>	5.15±0.06 <sup>a g h</sup>	2.63±0.15 <sup>a b d e g h</sup>
A	3.61±0.03 <sup>e g h</sup>	5.35±0.12 <sup>g h</sup>	5.31±0.05 <sup>a g h</sup>	3.09±0.05 <sup>a b g h</sup>
Lactic acid bacteria				
25 °C				
C	4.25±0.03 <sup>e h</sup>	7.48±0.00	7.94±0.08	8.72±0.02
P	4.37±0.05 <sup>e f h</sup>	7.48±0.00	7.37±0.01 <sup>a d h</sup>	8.42±0.12
E	4.09±0.01 <sup>b h</sup>	6.48±0 <sup>a b d</sup>	7.36±0.002 <sup>a d h</sup>	8.57±0.09
A	4.25±0.005 <sup>e h</sup>	7.48±0.00	8.17±0.02	8.44±0.04
Total Aerobic Mesophiles 25 °C				
C	5.61±0.01	7.48±0.00	8.06±0.00	8.64±0.05
P	5.84±0.08	7.48±0.00	8.00±0.05	8.37±0.06
E	4.96±0.002 <sup>a b d</sup>	6.48±0.00 <sup>a b d</sup>	7.82±0.03 <sup>a</sup>	8.46±0.06

A	5.33±0.03 <sup>ab</sup>	7.48±0.00	8.01±0.05	8.35±0.04
<i>Enterobacteriaceae</i>				
4 °C				
C	5.37±0.02 <sup>h</sup>	5.05±0.05	5.49±0.01 <sup>gh</sup>	6.85±0.03 <sup>ghi</sup>
P	5.29±0.03 <sup>h</sup>	4.76±0.02 <sup>gh</sup>	5.53±0.04 <sup>gh</sup>	5.57±0.08 <sup>acghi</sup>
E	4.11±0.01 <sup>abdh</sup>	4.39±0.01 <sup>agh</sup>	5.30±0.04 <sup>bdhg</sup>	6.03±0.01 <sup>agh</sup>
A	5.25±0.04	4.74±0.14 <sup>gh</sup>	5.61±0.04 <sup>h</sup>	4.45±0.04 <sup>abcgh</sup>
<i>Bacillus cereus</i>				
Agar 4 °C				
C	3.60±0.03 <sup>egh</sup>	3.17±0.06 <sup>eghi</sup>	3.78±0.04 <sup>eghi</sup>	2.26±0.08 <sup>ehi</sup>
P	5.05±0.01 <sup>ae h</sup>	3.95±0.05 <sup>ae ghi</sup>	3.71±0.03 <sup>eghi</sup>	2.27±0.04 <sup>dehi</sup>
E	3.61±0.01 <sup>begh</sup>	3.26±0.06 <sup>beghi</sup>	3.61±0.04 <sup>eghi</sup>	2.41±0.08 <sup>deh</sup>
A	3.61±0.03 <sup>begh</sup>	3.17±0.13 <sup>beghi</sup>	3.58±0.05 <sup>eghi</sup>	2.85±0.04 <sup>ae h</sup>
Lactic acid bacteria				
4 °C				
C	4.25±0.03 <sup>eh</sup>	5.36±0.06 <sup>i</sup>	6.05±0.01 <sup>i</sup>	7.36±0.005 <sup>hi</sup>
P	4.37±0.05 <sup>efh</sup>	5.16±0.03 <sup>hi</sup>	5.87±0.01 <sup>ahi</sup>	7.29±0.02 <sup>dhi</sup>
E	4.09±0.01 <sup>bh</sup>	5.14±0.04 <sup>i</sup>	5.66±0.02 <sup>abhi</sup>	7.43±0.01 <sup>hi</sup>
A	4.25±0.00 <sup>e</sup>	5.08±0.02 <sup>ai</sup>	5.61±0.03 <sup>abhi</sup>	7.39±0.02 <sup>hi</sup>
Total Aerobic				
Mesophiles 4 °C				
C	5.61±0.01	5.39±0.03 <sup>i</sup>	6.17±0.03 <sup>i</sup>	7.64±0.10 <sup>i</sup>
P	5.84±0.08	6.10±0.04 <sup>ai</sup>	6.44±0.03 <sup>ci</sup>	7.36±0.02 <sup>di</sup>
E	4.96±0.002 <sup>abd</sup>	5.26±0.02 <sup>bi</sup>	6.02±0.07 <sup>bi</sup>	7.32±0.08 <sup>di</sup>
A	5.33±0.03 <sup>ab</sup>	5.10±0.03 <sup>abi</sup>	5.99±0.03 <sup>bi</sup>	8.02±0.02 <sup>ai</sup>

Note: T, incubation time (days). Statistical significance ( $p < 0.05$ ):<sup>a</sup>, significant differences from control; <sup>b</sup>, significant differences from sample with *Paenibacillus* inoculated (P); <sup>c</sup>, significant differences from treatment E; <sup>d</sup>, significant differences from treatment A (same temperature, same media); <sup>e</sup>, significant differences from media EMB; <sup>f</sup>, significant differences from media *Bacillus cereus* agar; <sup>g</sup>, significant differences from media MRS; <sup>h</sup>, significant differences from media TSA (same temperature, same treatment); <sup>i</sup>, significant differences from different temperature (same media, same treatment).

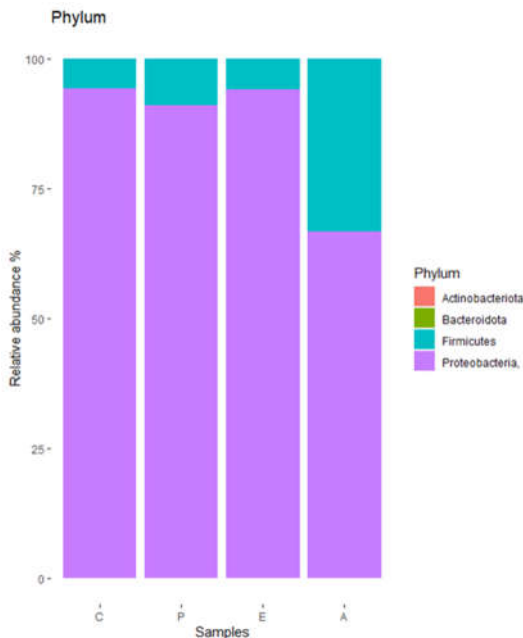
### 3.3 Bacterial biodiversity

The numbers of assigned reads and alpha diversity indices of controls and treated samples are shown in Table 2. The indices show that there was not a great diversity of species and there was dominance of a low number of species.

**Table 2.** Number of readings and alpha diversity indices at the genus level.

Sample	Nº Reads	Chao1	Shannon	Simpson
C1	123226	25	1.703	0.749
C2	119676	30	1.849	0.777
C3	127885	29	1.724	0.739
P1	130156	25	1.532	0.702
P2	112884	28	1.885	0.787
P3	130114	28	2.050	0.822
E1	124532	28	1.224	0.494
E2	113295	27	1.422	0.626
E3	119261	26	1.814	0.787
A1	125674	26	2.056	0.822
A2	121055	29	1.988	0.812
A3	122216	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).

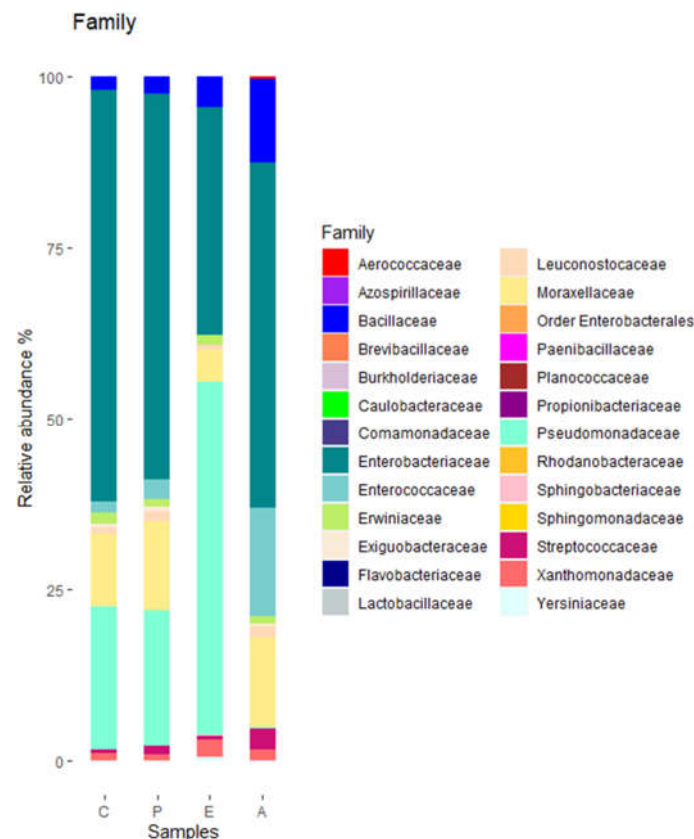


**Figure 1.** Bacterial diversity of puree samples at phylum level. C, untreated controls. P, puree inoculated with *Paenibacillus dendritiformis* UJA2219. E, puree treated with partially-purified extract (1.5 M eluate). A, puree treated with partially-purified extract (60% acetonitrile).

The different operational taxonomic units (OTU) found in the puree samples were grouped into 4 phyla (Figure 1), of which *Proteobacteria* were by far the main representatives, followed by

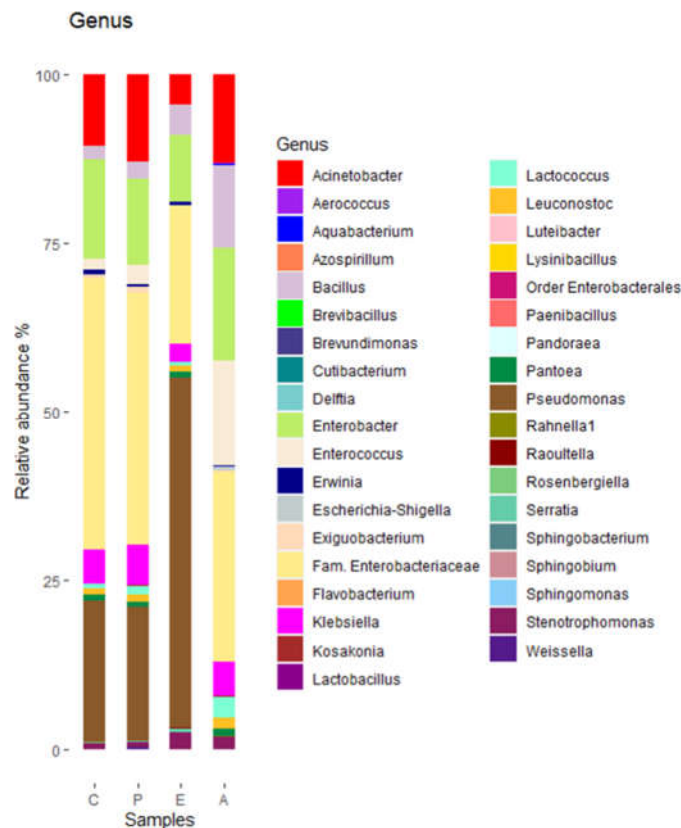
*Firmicutes*. Remarkably, samples corresponding to treatment A showed a lower relative abundance of *Proteobacteria* and a higher relative abundance of *Firmicutes*.

These phyla included representatives of 26 families. Of them, 8 families had relative abundances of at least 2% (Figure 2). *Pseudomonadaceae* and *Enterobacteriaceae* were the families with higher relative abundances. The relative abundance of *Pseudomonadaceae* was highest in sample E and lowest in sample A. At the genus level, a total of 37 genera were detected. Of them, 10 had relative abundances of at least 2% (Figure 3).

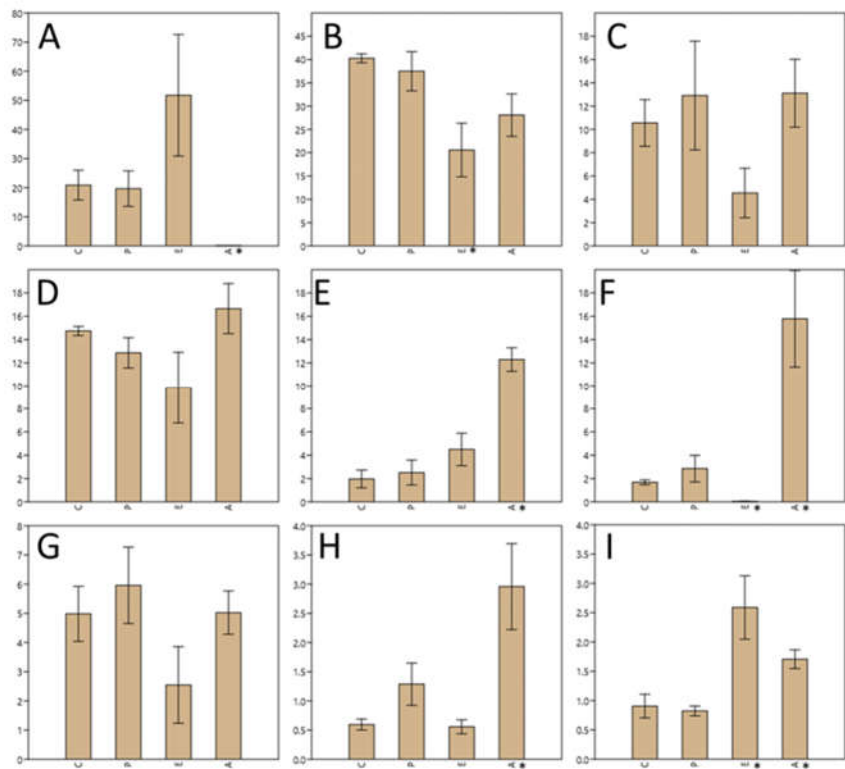


**Figure 2.** Bacterial diversity of puree samples at family level. C, untreated controls. P, puree inoculated with *Paenibacillus dendritiformis* UJA2219. E, puree treated with partially-purified extract (1.5 M eluate). A, puree treated with partially-purified extract (60% acetonitrile).





**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).



**Figure 4.** 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  $\pm$  standard deviation).



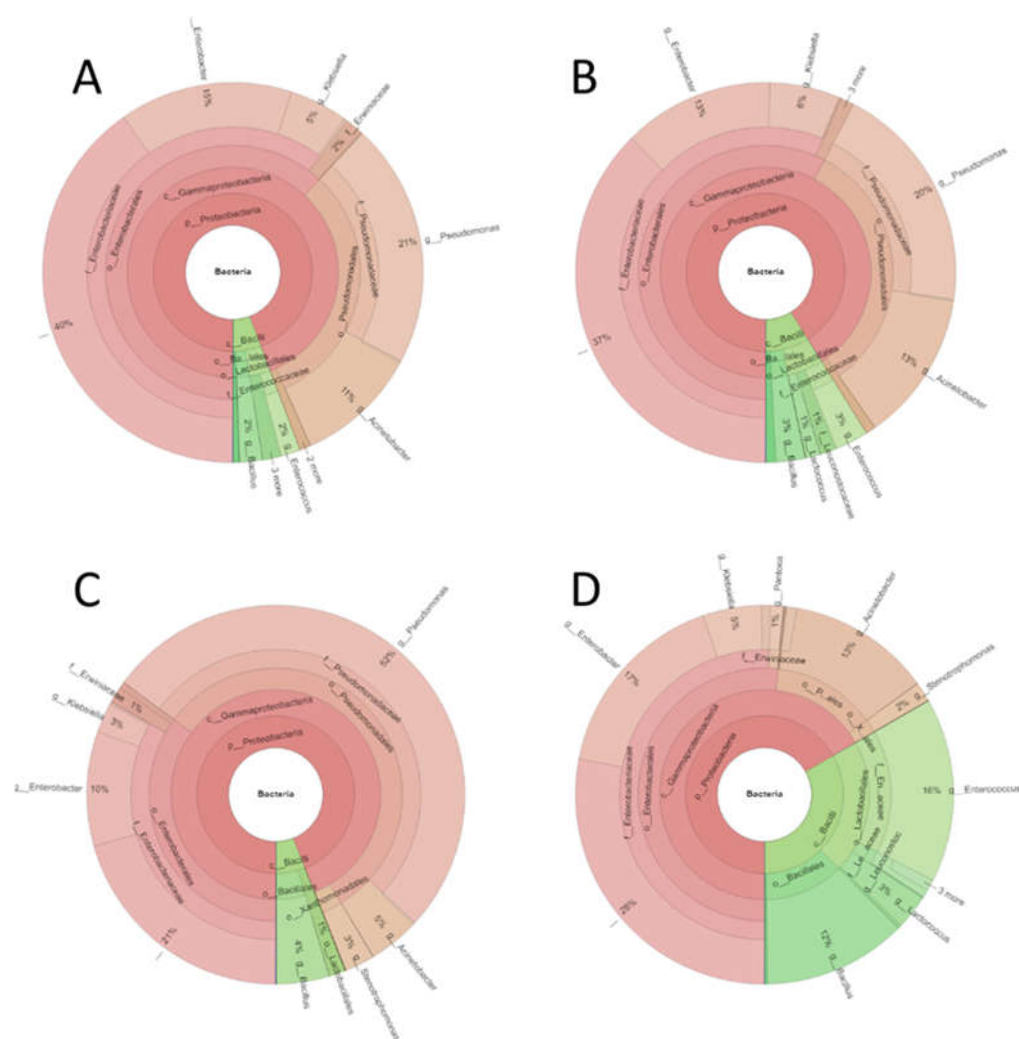
deviation). X axis indicates sample type (C, controls; P, treatment with *Paenibacillus* strain; E, treatment with NaCl eluate; A, treatment with acetonitrile eluate. Asterisk denotes statistically significant ( $p < 0.05$ ) differences with controls. Each graphic represents a different genus or taxonomic group: A, *Pseudomonas*; B, fam. *Enterobacteriaceae*; C, *Acinetobacter*; D, *Enterobacter*; E, *Bacillus*; F, *Enterococcus*; G, *Klebsiella*; H, *Lactococcus*; I, *Stenotrophomonas*.

Figure 4 shows the most representative genera according to the relative abundance in the different samples, with the *Enterobacteriaceae* family presenting 40% relative abundance 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 a quite similar percentage both in the control and in the sample inoculated with *Paenibacillus* (around 20%), 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.

Genus *Acinetobacter* and *Enterobacter*, which represented between 10% and 14% of the total OTUs, treatment with NaCl eluate reduced the population by at least half. Genus *Acinetobacter* represents 10% of the population, this percentage increased after *Paenibacillus* inoculation to about 13% and remained so after treatment with acetonitrile eluate, while it decreased to 4.5% when treated with NaCl eluate.

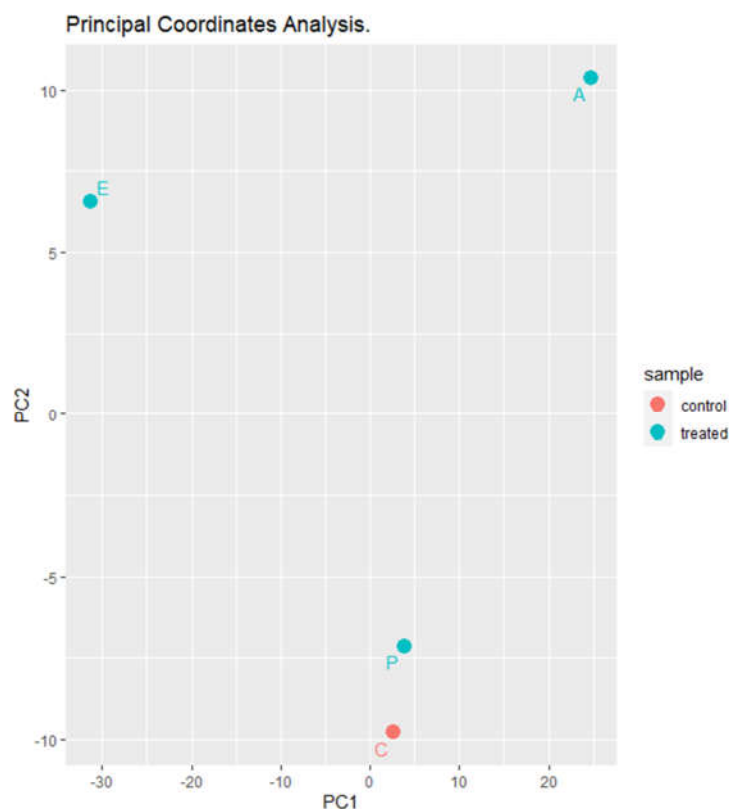
The treatment with the acetonitrile eluate (A) significantly ( $p < 0.05$ ) increased the relative abundance of *Bacillus*, *Enterococcus* and *Lactococcus*. Treatment with NaCl eluate significantly ( $p < 0.05$ ) decreased the population of *Enterococcus* while significantly ( $p < 0.05$ ) increased that of *Stenotrophomonas*.

Figure 5 shows the biodiversity of the different samples represented using the Krona tool.



**Figure 5.** Comparison of the means different biodiversity percentages using Krona Tools. A, untreated controls. B, puree inoculated with *Paenibacillus dendritiformis* UJA2219. C, puree treted with partially-purified extract (1.5 M eluate). D, puree treted with partially-purified extract (60% acetonitrile). .

Principal coordinate analysis (PCoA) (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*. 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).

#### 4. Discussion

The results obtained in the present study indicate that *Paenibacillus dendritiformis* UJA2219 produces antimicrobial activity against bacteria of 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.

*Paenibacillus dendritiformis* is described to produce antimicrobial peptides [18]. These peptides are being studied for their antifungal effect to prevent crop colonization by fungi [19,20]. 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 like paenibacterin, pelgipectin and paenilamicin. It could be that the addition of *Paenibacillus 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 like pH, aeration, incubation temperature, time of incubation, available nutrients, the food matrix, and the competing microbiota [21].

The treatments carried out with partially-purified preparations from *P. dendriformis* UJA2219 cultured broths exerted an antimicrobial effect on the vegetable puree incubated at 4 °C and 25 °C that depended on the microbial group. Although this activity was more pronounced at 25 °C than under refrigerated conditions, it helps 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 this was no longer observed after 7 days; for presumptive *Bacillus*, an efficacy of the two eluates (A and E) was observed after 7 days, and in presumptive *Enterobacteriaceae* there was a great reduction in both the sample inoculated with *Paenibacillus* and the eluate (E), and even the CFU/ml levels decreased below the detection limits after 7 days with eluate (A). On the other hand, at 4 °C, there were no differences with respect to the control sample in total aerobic mesophilic counts, presumptive lactic acid bacteria or *Bacillus*, but we did observe a decrease in the number of presumptive *Enterobacteriaceae* with the treatments applied.

In published studies of the efficacy of *Paenibacillus* as an antibacterial 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 [22]. *Paenibacillus* has also been shown to be effective against other bacterial groups such as *Clostridium* or *E. coli* [23]. Most of these studies are based on assays performed with the strain in question, not with the purified fractions. The present study shows that the production of these metabolites and their subsequent purification could be used as preservatives in this food model.

Regarding the impact of *Paenibacillus* and its partially-purified extracts on bacterial diversity, the results we have obtained so far indicate that inoculation with 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 microbial proliferation. Regarding treatments with culture extracts, for the most representative genera of the samples such as *Pseudomonas* and *Enterobacteriaceae* the observed effect 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, we should take into account that the selective medium used (EMB) also supports growth of *Pseudomonas* 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*.

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. Many of these peptides are cationic and could be recovered from culture supernatants by cation exchange. Others, however, may be amphipathic or hydrophobic (as in the case of lipopeptides) and show a low solubility in the water used as eluant 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 bacterial diversity. Furthermore, the eluant itself may also have an effect on the peptide solubility and final activity. We must also keep in mind that 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 *Paenibacillus dendritiformis* UJA2219. Further experiments with the purified peptides are needed in order to understand better their impact on the bacterial communities of food systems and their potential as new biopreservatives.

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