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Research article

Determination of the effect of the bacteriocin enterocin AS-48 on the microbial loads and bacterial diversity of blueberries

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ABSTRACT

Fresh fruits have been involved in transmission of foodborne pathogens. In the present work, five different batches of blueberries were used. One aliquot from each batch was washed with sterile saline solution (SSS) and the other one with a solution of the circular bacteriocin enterocin AS-48 in SSS. Then, the surface microbiota of controls and bacteriocin-treated samples was recovered and used for microbiota analyses, both using viable counts and high-throughput amplicon sequencing. Total aerobic mesophilic loads ranged from 2.70 to 4.09 log CFU/g in most of the samples. Only two samples yielded detectable viable counts on selective media (*Enterobacteriaceae*, presumptive *Salmonella* and coliforms), with values ranging from 2.84 to 3.81 log CFU/g. The bacteriocin treatment reduced viable cell counts of total aerobic mesophiles to a range of 1.40–1.88 log CFU/g. No viable cells were detected on selective media. Amplicon sequencing indicated large batch-to-batch variations in the surface microbiota of blueberries and also an effect of the bacteriocin treatment on microbiota composition.

1. Introduction

Blueberries (*Vaccinium myrtillus*) are rich in bioactive compounds, represented mainly by polyphenols of the flavonoid type, especially anthocyanidins and flavonols [1], as well as antioxidants, such as vitamin C, B complex, E and A [2], and phenolic acids, particularly hydroxycinnamic acids [3]. All these components are closely related to the numerous health-promoting properties attributed to plant-based foods. This has led to increased public interest in plant-based foods, resulting in a large increase in their consumption [4–6].

Fresh fruits, including blueberries, have been implicated in the transmission of foodborne pathogens [7]. Several outbreaks associated with the consumption of blueberries have been reported [8–10]. Recently, a recall of a batch of frozen blueberries contaminated with *Listeria monocytogenes* has been reported [11]. For this reason, it is essential to carry out treatments that allow us to maintain the nutritional and organoleptic properties of the product, while guaranteeing its food safety and shelf life extension. Among the different food biopreservation methods used in the food industry are bacteriocin treatments [12]. Bacteriocins are ribosomal antimicrobial peptides produced by certain bacterial strains, which can be degraded by proteolytic enzymes in the gastrointestinal tract of mammals and can therefore be considered safe food biopreservatives [13]. The bacteriocin chosen for this study was enterocin

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AS-48, which is a cyclic bacteriocin of class IIc produced by *E. faecalis* and which has bactericidal activity against a large number of Gram-positive bacteria of importance in food, such as *Listeria*, *Bacillus* and *Clostridium* sp [14,15]. This bacteriocin has been extensively studied in our research group in recent years, so that we can confirm its high stability at acid pH and high temperatures, which gives it a high potential to be used for the treatment of various types of food.

Application of next-generation sequencing technologies can provide novel data on the effects of bacteriocins in food systems. This study aimed at providing information on the impact of enterocin AS-48 treatments on the bacterial diversity of blueberries.

2. Materials and methods

2.1. Preparation of blueberries samples

Five batches of blueberries purchased in bulk from five different fruit shops just before processing were used. From each batch of blueberries, 2 samples of 10 g each were prepared, one of which was intended to be treated with bacteriocin (designated as "AB" samples), and the other as a control ("A" samples). Finally, a total of 10 samples were obtained, of which 5 were controls and the remaining 5 bacteriocin-treated. In addition, the different samples were packaged in sterile 50-mL propylene Falcon tubes for further processing.

2.2. Enterocin AS-48 treatment

The bacteriocin treatment consisted of immersing the blueberries in a solution of Enterocin AS-48 (50 μg/mL) for 10 min (or in an equivalent sterile saline solution lacking bacteriocin for control samples). The bacteriocin preparation by cation exchange chromatography followed by dialysis through benzoylated cellulose tubing (MW cutoff, 2000; Sigma) against sterile saline solution as described elsewhere [16]. The bacteriocin preparation was cleaned through low protein binding filters (0.22 μm pore size, Millex GV; Millipore Corp., Belford, Mass., U.S.A.) under sterile conditions before use. After treatment, the entire volume of bacteriocin solution poured over the 5 treated samples was decanted and the samples were left to dry in their containers for 1 h at room temperature. Each sample was treated with a different aliquot of the bacteriocin solution. All samples (treated or untreated) were then stored refrigerated at 4 °C for 24 h. After storage, the samples were washed separately on the surface with 10 mL of sterile saline solution under gentle stirring for 10 min, in order to recover surface microbiota.

2.3. Microbiological analysis

For each microbial cell suspension collected from each sample, an aliquot (2 mL) was serially diluted by using sterile saline solution. The serial dilutions were plated in triplicate on trypticase soya agar (TSA) for total aerobic mesophiles, MacConkey agar for presumptive *Enterobacteriaceae*, Eosine Methylene Blue Agar (EMB Agar) for coliforms and on Brilliant Green Agar (BGA) for *Salmonella*. The incubation period was 24 h at 37 °C for all culture media.

2.4. DNA extraction

The remaining volume of the solution were centrifuged at 600×*g* for 5 min in order to remove solids. An aliquot (1.5 mL) of the resulting supernatant was centrifuged (13,500 g, 5 min) to recover microbial cells. After centrifugation, the supernatant was discarded and the pellets were used for DNA extraction with a DNeasy PowerSoil Kit (Qiagen, Madrid, Spain). The concentration and quality of the DNA was determined by QuantiFluor® ONE dsDNA system (Promega, Madison, WI, USA).

2.5. DNA sequencing and analysis

Library preparation was carried out according to the Illumina Metagenomic Sequencing Library Preparation protocol (provided by Illumina, Inc., San Diego, CA, USA), targeting the V3–V4 regions of the 16S rRNA gene. Illumina adapter overhang nucleotide sequences were added to the gene-specific sequences. The modified PCR primers were: forward primer: 5′TCGTCGGCA GCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG; and reverse primer: 5′GTCTCGTGGGCTCGGAGATGTGTATAAGA GACAGGACTACHVGGGTATCTAATCC [17]. The protocol was initiated starting from microbial geomic DNA (5 ng/μL in 10 mM Tris pH 8.5). After 16S rRNA gene amplification, the multiplexing step was performed using Nextera XT Index Kit (Illumina). The size of PCR products (expected size ~550 bp) was verified with a Bioanalyzer DNA 1000 chip. Libraries were sequenced using a 2 × 300 pb paired-end run (MiSeq Sequencer, Illumina). Quality assessment was performed by the use of prinseq-lite program [18]. The sequence data were analyzed using qiime2 pipeline [19]. Denoising, paired-ends joining, and chimera depletion was performed starting from paired ends data using DADA2 pipeline [20]. Taxonomic assignment was carried out using the Naive Bayesian classifier integrated in qiime2 plugins and the SILVA_release_132 database [21]. Statistical analysis was carried out with SPSS software v. 24 (IBM Corp., Foster City, CA, USA).

2.6. Statistical analysis

Viable cell count data were analyzed by one-way ANOVA and Tukey's test. Data from the bacterial diversity study were compared

by Principal Coordinates Analysis.

3. Results

3.1. Effect of enterocin AS-48 treatments on microbial loads

Overall, all enterocin AS-48 treatments significantly reduced viable counts compared to untreated control samples (Table 1).

As for the counts of total aerobic mesophiles carried out on TSA, we found that the bacteriocin-treated samples showed viable counts between 1.4 and 1.88 log CFU/g, significantly lower ($p < 0.05$) compared with the control samples without bacteriocin, whose range of count values was between 2.7 and 4.09 log CFU/g. Furthermore, regardless of the treatment used, two of the samples (A1-A1B and A4-A4B) had counts below the detection limit of 1.0 log CFU/g (Table 1).

The counts of presumptive *Enterobacteriaceae*, obtained on MacConkey agar (MC), were similar for two of the control samples, with values of 3 and 3.23 log CFU/g for samples A3 and A2 respectively. For the remaining control samples and all treated samples, counts below the detection limit were obtained on this culture medium (Table 1).

In the case of presumptive *Salmonella* counts, carried out on Brilliant Green Agar (BGA), significant counts were only obtained in two control samples, A2 and A3. Sample A2 showed a value of 3.81 log CFU/g, which was significantly higher ($p < 0.05$) compared to sample A3, which had a value of 2.84 log CFU/g. Similar results were obtained in the counts carried out on Eosin-Methylene Blue Agar (EMB), for coliforms, being in this case the viable cell values of 3.61 and 3.04 log CFU/g for the control samples A2 and A3, respectively, also finding significant differences ($p < 0.05$). For the rest of the control samples and all the treated samples, counts were below the detection limit in both culture media (Table 1).

3.2. Bacterial diversity

The total numbers of assigned reads ranged from 18,587 to 54,911 (Table 2). However, a percentage ranging from 74.38 to 97.20% of the reads/sample was assigned to chloroplast and mitochondria and therefore removed before further analysis. Cleaned data were used to calculate diversity indices and taxonomic microbiota composition. The Chao-1, Shannon and Simpson diversity indices obtained at genus level are reported in Table 2.

The relative abundance values of the different Amplicon Sequence Variants (ASVs) found on the blueberry samples are shown in Fig. 1. The results obtained indicated the presence of 16 phyla (Fig. 1A).

In the control samples, *Firmicutes* had relative abundances of 56.39% in sample A1 and of 45.13 in sample A4. The following relative abundances were found for *Proteobacteria* in control samples A2 (96.30%), A3 (89.41%) and A5 (45.88%). *Actinobacteriota* had relative abundances of 11.55% (sample A1), 13.30% (sample A4) and 16.80% (sample A5). As shown in Fig. 1B, the *Firmicutes* group was mainly represented by members of the *Enterococcaceae* family (mainly in samples A1 and A4, with the genus *Enterococcus*), *Bacillaceae* family (sample A1, genus *Bacillus*) and *Staphylococcaceae* (sample A5, genus *Staphylococcus*). In the *Proteobacteria* group, the *Enterobacteriaceae* family had relative abundances of 90.57% (A2) and 71.63% (A3). In sample A3, genus *Erwinia* had a relative abundance of 55.70% (Fig. 1C).

Bacteriocin-treated samples showed the highest relative abundances of *Proteobacteria*, with the exception of sample A5B (Fig. 1A). In sample A1B, the most important families detected were *Enterobacteriaceae* (40.84%), *Phyllobacteriaceae* (14.01%, mainly represented by the genus *Parvibaculum*), *Hyphomicrobiaceae* (12.88%) and *Xanthomonadaceae* (7.56%), while the families *Enterococcaceae* and *Bacillaceae* decreased below 2.6% in relative abundance compared to the corresponding control sample A1 (Fig. 1B). A slight decrease in the relative abundance of *Sphingomonadaceae* was also detected. In sample A2B, the only notable change from the control A2 was a slight increase in the relative abundance of *Oxalobacteraceae* (2.58%) and a decrease in *Pseudomonadaceae*. In sample A3B, the increase of *Proteobacteria* detected compared to the control (from 89.41 to 95.18%) was mainly due to an increase of *Enterobacteriaceae*, reaching 89.69%, and to a much lesser extent of *Legionellaceae* (2.45%, represented by the genus *Legionella*). In contrast, the

Table 1
Viable cell counts in blueberry samples treated or not with enterocin AS-48.

Sample	TSA	MC	BGA	EMB
A1	<1.00	<1.00	<1.00	<1.00
A2	4.09 ± 0.21[a]	3.23 ± 0.07	3.81 ± 0.01[b]	3.61 ± 0.02[c]
A3	3.65 ± 0.25[a]	3.00 ± 0.03	2.84 ± 0.30	3.04 ± 0.01
A4	<1.00	<1.00	<1.00	<1.00
A5	2.70 ± 0.03[a]	<1.00	<1.00	<1.00
A1B	<1.00	<1.00	<1.00	<1.00
A2B	1.40 ± 0.12[a]	<1.00	<1.00	<1.00
A3B	1.48 ± 0.21[a]	<1.00	<1.00	<1.00
A4B	<1.00	<1.00	<1.00	<1.00
A5B	1.88 ± 0.21[a]	<1.00	<1.00	<1.00

Samples treated with enterocin AS-48 are indicated with "B". The remaining samples (without "B") correspond to the untreated control samples. Each number corresponds to the 5 different batches of blueberries used in the trial. Statistical significance ($p < 0.05$): [a], significantly lower than the untreated control; [b], significantly higher than control sample A3 (in BGA);[c], significantly higher than control sample A3 (in EMB).

Table 2
Number of reads and alpha diversity indices at genus level of blueberry samples, treated and not with enterocin AS-48.

Sample	*Total reads assigned*	*Reads assigned to prokaryotes*	*Chao-1*	*Shannon*	*Simpson*
A1	18,587	4761	80	3.32	0.91
A1B	46,576	7266	67	2.50	0.80
A2	49,922	8332	43	0.58	0.18
A2B	54,867	11,336	44	0.67	0.22
A3	43,194	5206	51	2.00	0.66
A3B	48,197	11,820	46	0.75	0.26
A4	51,473	3601	84	3–36	0.92
A4B	49,697	2132	45	2.96	0.88
A5	23,922	668	32	3.19	0.95
A5B	54,911	2632	39	2.03	0.65

Samples treated with enterocin AS-48 are indicated with "B", while the others are untreated controls. Each number corresponds to the 5 different batches of blueberries analyzed in the trial.

percentage of reads assigned to the genus *Erwinia* decreased compared to the control (from 55.70% to 3.76%). For sample A4B, the increase in the relative abundance of *Proteobacteria* was much higher (from 33.65% to 71.63%). However, in this case, besides the *Enterobacteriaceae* family, which reached 30.72%, the following other families had slightly higher relative abundances: *Hyphomicrobiaceae* (6.70%), *Phyllobacteriaceae* (6.19%), *Legionellaceae* (3.51%, represented by the genus *Legionella*), *Pseudomonadaceae* (3.38%; genus *Pseudomonas*), *Xanthomonadaceae* (5.30%), and Order ASSO-13 (3.05%). Only in sample A5B we observed an increase in the relative abundance of *Firmicutes* after bacteriocin treatment. This group reached 63.22% and was mainly represented by the *Enterococcaceae* family with 58.62%. In contrast, representatives of *Actinobacteria* and *Proteobacteria* decreased in relative abundances (to 4.14 and 29.26%, respectively). In *Proteobacteria*, the groups that decreased the most in relative abundance were the families *Hyphomicrobiaceae* (genus *Parvibaculum*), *Sphingomonadaceae* (genus *Sphingomonas*), *Haliangiaceae*, *Enterobacteriaceae* and the order *Burkholderiales*. In contrast, the relative abundances of *Pseudomonadaceae* and *Xanthomonadaceae* increased compared to the control sample A5.

Principal Coordinates Analysis (Fig. 2) indicated a closer proximity for bacteriocin-treated samples corresponding to batches 1–4, which clustered separately from the control samples (except sample A2). On the other hand, the control samples showed a more dispersed distribution (except for samples A1 and A4, which were also very close to the treated sample A5B), and samples A3 and A5 which showed positions further away from all the others.

4. Discussion

The results for the five different batches of blueberries purchased from different supermarkets showed that three of them had total aerobic mesophilic counts between 2.7 and 4 log CFU/g, and that the two samples showing higher counts also had considerable levels of coliforms and suspected *Salmonella* according to the counts obtained on selective media. These results could be considered alarming, considering the risks of contamination and infectious outbreaks mentioned above.

In order to reduce the microbial load and avoid the risk of spreading pathogens, different methods have been tested, such as washing with solutions containing different types of disinfectants or biocidal products. Biological methods, such as the use of bacteriocins, are better accepted by consumers compared to chemically synthesised products. Previous studies carried out with enterocin AS-48 reinforce the value of this bacteriocin as a potential food biopreservative [22–26]. The results obtained in the present study indicated that washing with a bacteriocin solution significantly reduced the total mesophilic aerobic counts in the three samples that initially showed elevated values for this group and, more importantly, reduced the enteric bacteria counts below the detection limit in the two samples that had tested positive on selective media for this type of bacteria. Numerous previous studies have shown that AS-48 acts on the cytoplasmic membrane and that Gram-negative bacteria show much higher levels of resistance because their outer membrane acts as a barrier to the diffusion of the bacteriocin to its primary target (the cytoplasmic membrane). However, it has also been shown that any agent that alters the permeability of the outer membrane increases the sensitivity of Gram-negative bacteria to this bacteriocin. Blueberries contain different types of phenolic compounds with marked antibacterial activity. In particular, more than 15 types of anthocyanins (which are mainly present in the skin and are soluble in water) have been described which, in addition to their bactericidal activity against pathogenic bacteria, they also show an outstanding ability to inhibit bacterial adhesion and biofilm formation [27]. Therefore, it could be speculated that the observed decrease in counts is due to the combined effect of bacteriocin and antimicrobial compounds in blueberries either by facilitating bacterial inactivation or by decreasing bacterial adherence (thus facilitating their removal by washing) or both. It should also be noted that AS-48 is an amphipathic molecule (i.e. it has a strongly positively charged region as well as hydrophobic regions), so it could also have some detergent action. Another possibility to consider is a possible sublethal effect by the combination of the antimicrobials present in the fruit and AS-48, which would prevent the recovery of damaged bacterial cells on selective media [28]. Finally, a previous study carried out in our laboratory showed that bacteriocin washing significantly reduced or eliminated *L. monocytogenes* in a variety of fruits such as strawberries, raspberries and blackberries [24]. The lower survival of the bacteria on these substrates and the greater effect of bacteriocin compared to other fruits were attributed to the more acidic pH of the former.

Another outstanding aspect of the present study is the differences in the bacterial biodiversity of the samples analyzed. Most of the

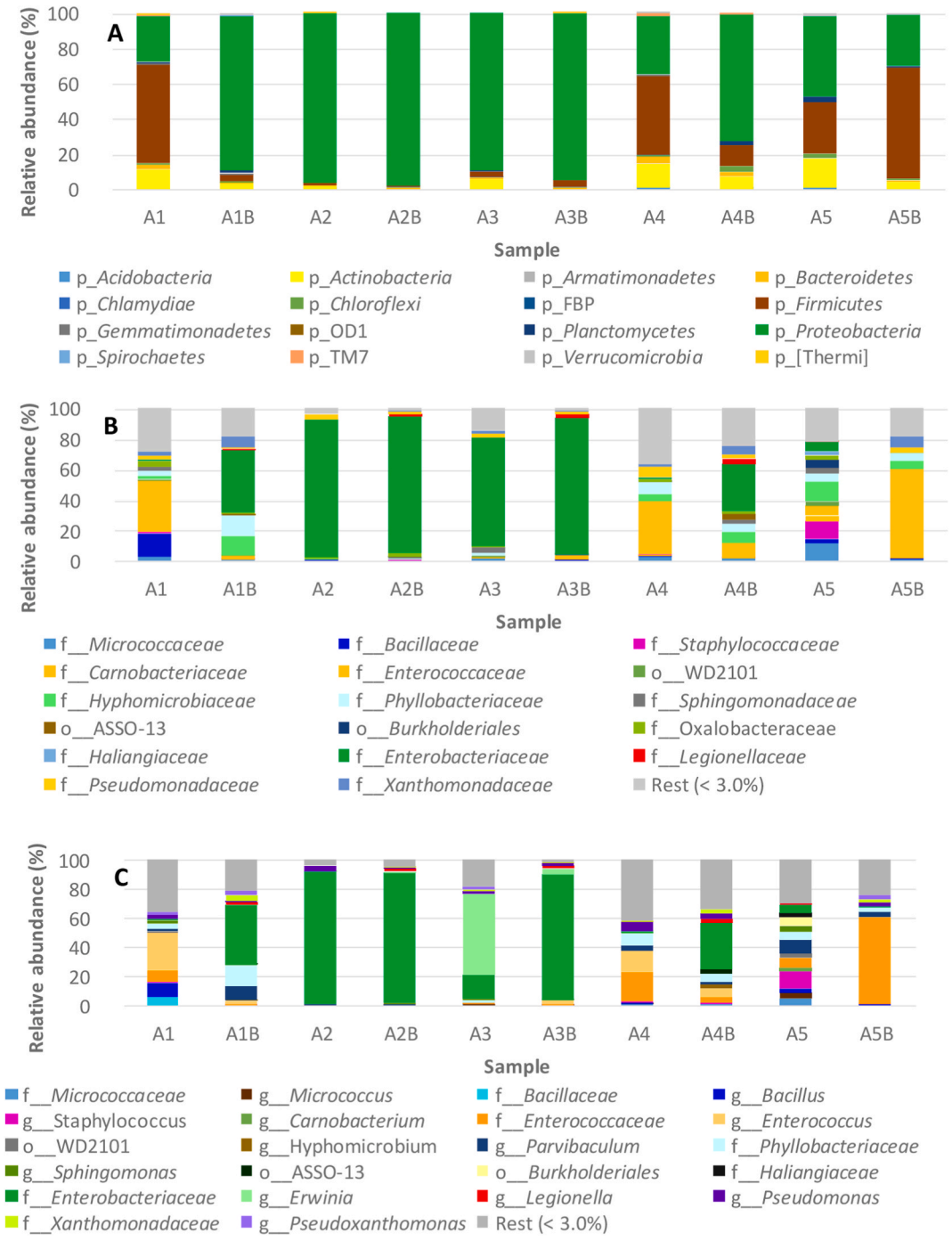

Fig. 1. Bacterial diversity of blueberry samples at phylum (A), family (B) and genus (C) level. Samples treated with enterocin AS-48 are indicated with "B". The remaining samples (without "B") correspond to the untreated control samples. Each number corresponds to the 5 different batches of blueberries used in the trial.

assigned reads belonged to chloroplasts (and to a less extent to mitochondria). This could be attributed to degradation of the fruit tissue (possibly because of a prolonged storage after harvest) and contamination with juice from the fruit. This could also explain why the numbers of reads assigned to prokaryotes were much lower than expected. Even if the number of reads obtained for prokaryotes was low, there seemed to be clear differences between samples. Samples A2 and A3 were characterised by a higher relative abundance of *Proteobacteria*, mainly of the family *Enterobacteriaceae*, while in the rest of the samples *Firmicutes* had a much higher relative abundance. These results are in agreement with those obtained on selective media for samples A2 and A3. The differences found between

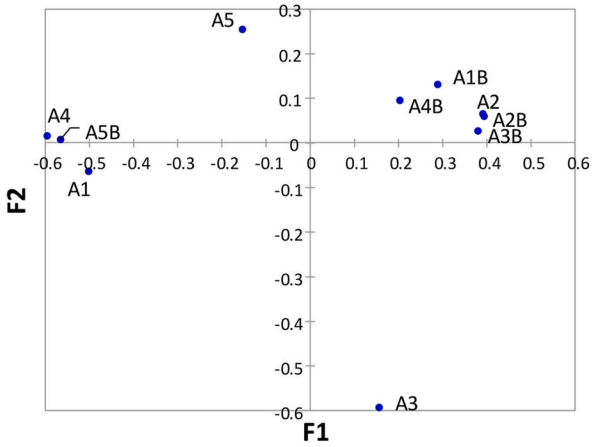

Fig. 2. Principal coordinate analysis (PCoA) of blueberry samples treated or not with enterocin AS-48. Samples treated are indicated with "B". The remaining samples (without "B") correspond to the untreated control samples. Each number corresponds to the 5 different batches of blueberries used in the trial.

samples could be due to several factors: origin, cultivation practices, processing and storage time. It has been reported that the conditions of the growing environment, especially the presence of animal farms, have a considerable influence on the microbiota associated with fruits and vegetables. This could be an explanation for the higher relative abundance of *Enterobacteriaceae* in two of the samples, although the origin of the samples was unknown. However, among the *Firmicutes*, some of the bacterial groups detected (such as *Staphylococcus* or *Enterococococcus*) could be linked to farms, contaminated water, or to the handling itself (in this case being of human origin). *Enterococcus* has also been isolated from soil, with some species being typically epiphytic, while others can be transmitted from one environment to another through the air or by vectors such as insects. As for the rest of the microbial groups detected, in most cases they are bacteria typically associated with plants (such as *Xanthomonadaceae*, *Phyllobacteriaceae*, *Pseudomonas*, *Sphingomonas* or *Erwinia*). Most of them are saprophytic bacteria that could play an important role in fruit deterioration.

In the samples washed with bacteriocin, the most significant changes were detected in those where the *Firmicutes* group had high relative abundances initially. This could be due to the fact that, according to previous studies, Gram-positive bacteria are much more sensitive to AS-48 compared to Gram-negative bacteria. Illustrative examples are the decrease observed in the relative abundance of *Bacillaceae*, *Bacillus*, *Enterococcaceae* and *Enterococcus* in sample A1B compared to control sample A1, the decrease in relative abundances of *Enterococcaceae* and *Enterococcus* in sample A4B compared to sample A4, and the decrease of *Micrococococaceae*, *Micrococcus*, *Carnobacterium* and *Staphylococcus* in sample A5B compared to A5. Since AS-48 has both bactericidal and bacteriolytic effects, we would expect lysis of these Gram-positive bacteria and loss of their released DNA by degradation and washing steps. An important exception to these examples is the very marked increase in the relative abundance of *Enterococcaceae* in sample A5B compared to A5, when the opposite would be expected. This could be explained assuming the presence of enterococci carrying the enterocin AS-48 resistance genes in the samples, and whose presence could be enriched compared to the other microbial groups precisely because of the bacteriocin treatment. Several studies have demonstrated the production of enterocin AS-48 in enterococci of different origins [29]. There are also other circular bacteriocins [30], some of which may have a structure-function relationship equivalent to AS-48 and may carry genetic determinants capable of conferring cross-immunity with AS-48.

Author contribution statement

Javier Rodríguez López, M[a] José Grande Burgos: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper. Francesca De Filippis: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper. Rubén Pérez Pulido, Rosario Lucas: Conceived and designed the experiments; Performed the experiments. Danilo Ercolini: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data. Antonio Galvez: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Data availability statement

Data will be made available on request.

Declaration of interest's statement

The authors declare no competing interests.

Declaration of competing interest

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

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