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# Biofilms formed by microbiota recovered from fresh produce: Bacterial biodiversity, and inactivation by benzalkonium chloride and enterocin AS-48

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ABSTRACT

Microbiota recovered from fresh produce (Romaine lettuce, endives and cucumbers) was allowed to form biofilms on stainless steel coupons. The formed biofilms were treated with benzalkonium chloride (BC) at three different concentrations (0.01, 0.1, and 1.0 g/l), enterocin AS-48 (50 μg/ml) and combinations of BC and enterocin AS-48. The single treatment with bacteriocin had no effect on viability of sessile bacteria. A high concentration of BC (1.0 g/l) was required to achieve 4.1 logs reduction of viable cell counts. The combination of BC (1.0 g/l) and enterocin AS-48 reduced viable cell counts below detectable levels. High-throughput sequencing analysis revealed that the formed biofilms were composed mainly by *Proteobacteria* of the genera *Pseudomonas* and *Stenotrophomonas*. Treatments with enterocin AS-48 and BC at sub-inhibitory concentrations only induced minor changes in the relative abundance of the different bacterial groups associated with biofilms.



## 1. Introduction

Biofilms are surface-associated, three-dimensional multicellular structures whose integrity depends upon the extracellular matrix produced by their constituent bacterial cells (Branda, Vik, Friedman, & Kolter, 2005; Jefferson, 2004). Biofilm formation has serious implications in industrial, environmental, public health and medical situations (Gilbert, McBain, & Rickard, 2003; Hall-Stoodley, Costerton, & Stoodley, 2004). The occurrence of biofilms in food-processing environments can cause post-processing contamination leading to lowered shelf-life of products and contamination by pathogens such as *Escherichia coli* O157:H7, *Salmonella enterica* or *Listeria monocytogenes* (Jessen & Lammert, 2003; Yaron & Römling, 2014). Sessile micro-organisms are more difficult to mechanically remove from food-contact surfaces and are also more resistant to disinfectants compared with planktonic forms (Gilbert, Das, Jones, & Allison, 2001; Morton, Greenway, Gaylarde, & Surman, 1998; Van Houdt & Michiels, 2010). In the case of vegetable foods, there is little information concerning biofilm formation in food processing facilities or on food contact surfaces.

Among the different approaches proposed to inactivate sessile bacteria is the use of bacteriocins in combination with biocides. Enterocin AS-48 is one of the best studied bacteriocins. It is a cyclic antimicrobial peptide with broad spectrum of antibacterial activity against Gram positive bacteria. The molecular structure, mode of action and genetic determinants of this bacteriocin have been deciphered, and its effects on planktonic bacterial cells have been studied in different food systems (Abriouel, Lucas, Ben Omar, Valdivia, & Gálvez, 2010; Grande Burgos, Pérez Pulido, López Aguayo, Gálvez, & Lucas, 2014; Maqueda et al., 2004). Previous studies showed that enterocin AS-48 could improve the efficacy of biocides against planktonic and sessile *Listeria monocytogenes* (Caballero Gómez, Abriouel, Grande, Pérez Pulido, & Gálvez, 2012), *Bacillus cereus* (Caballero Gómez, Grande, Pérez Pulido, Abriouel, & Gálvez, 2013a), *Staphylococcus aureus* (Caballero Gómez, Abriouel, Grande, Pérez Pulido, & Gálvez, 2013b) and *Salmonella enterica* (Grande Burgos, Pérez Pulido, López Aguayo, Gálvez, & Lucas, 2012).

Biocides derived from quaternary ammonium compounds are widely used. Among them, benzalkonium chloride is a disinfectant and cationic surface active agent used for sanitation in food processing lines and surfaces in the food industry (Krysinski, Brown, & Marchisello, 1992; Kuda, Yano, & Kuda, 2008; Ueda & Kuwabara, 2007). The purpose of the present study was to determine biofilm

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formation by microbiota recovered from fresh produce, and to determine the efficacy of enterocin AS-48, benzalkonium chloride and combinations of both antimicrobials on inactivation of biofilm bacteria. Since there is also little information on the composition of bacterial biofilms from vegetable foods, the study also aimed at determining the bacterial diversity in the formed biofilms by using high-throughput sequencing technology.

## 2. Materials and methods

### 2.1. Preparation of bacterial suspensions

Romaine lettuce, endives and cucumbers were purchased at a local supermarket. All were fresh vegetables with no signs of spoilage. The outermost leaves of lettuce were removed and discarded. For each vegetable type, three pieces were cut in small pieces (approx. 3 cm long) with a sterile knife and mixed. The cut vegetables (200 g lettuce; 210 g endives; 183 g cucumber) were placed separately inside sterile stomacher bags and mixed each one with 200 ml sterile buffered peptone water (BPW, Panreac, Barcelona, Spain). Mixing was done by hand-rubbing for 5 min at ambient temperature. Then, the resulting BPW suspensions were removed from the stomacher bags and centrifuged (3.500×*g* for 30 min at 4 °C). The resulting sediments containing the microbial cells recovered from each vegetable food were resuspended in 10 ml BPW each, mixed together in one 50 ml test tube, and washed by centrifugation (3.500×*g* for 30 min, 4 °C) with 50 ml BPW. The resulting sediment was resuspended in 10 ml BPW and stored at 4 °C (for not more than 2 h) until use as inoculum for biofilm formation.

### 2.2. Antimicrobials

Enterocin AS-48 was obtained from cultured broths of the producer strain *Enterococcus faecalis* A-48-32 after concentration by cation exchange chromatography as described elsewhere (Abriouel, Valdivia, Martínez-Bueno, Maqueda, & Gálvez, 2003). Bacteriocin concentrates were filtered through 0.22 μm pore size low protein binding filters (Millex GV; Millipore Corp., Belford, MA, USA) under sterile conditions. Bacteriocin concentrates were diluted 20-fold in sterile saline solution (SS) or in biocide solutions in order to achieve the desired final bacteriocin concentration of 50 μg/ml. Benzalkonium chloride (BC) commercial solution (Sigma-Aldrich, Madrid, Spain) contained 50% (wt/v) of the active compound.

### 2.3. Biofilm formation and antimicrobial treatments

Stainless steel coupons (1.5 by 4.0 cm, type 304 with a no. 4 finish) were sonicated in distilled water for 2 min, immersed in 70% ethanol for 10 min, rinsed with sterile distilled water and then dried in a biosafety cabinet for 4 h and sterilized by autoclaving. Coupons were placed individually inside 50 ml sterile Falcon test tubes with conical bottom containing 20 ml BPW inoculated (1%, vol/vol) with the bacterial cell suspension obtained from vegetable foods as described above. Two replicates consisting of 25 tubes each were prepared. After 48 h incubation at 30 °C, the liquid was removed from test tubes, and the formed biofilms were washed twice with 20 ml BPW. Washing was carried out gently by immersion in order to avoid disturbing the formed biofilms.

Following the washing steps, 20 ml of SS (controls) or antimicrobial solutions were added to each of the 50 ml Falcon tubes containing coupons. The following antimicrobial solutions were added: enterocin AS-48 at 50 μg/ml in SS, BC at final concentrations of 0.01, 0.1, and 1.0 g/l in SS, or combinations of the above-mentioned BC solutions plus 50 μg/ml enterocin AS-48. Coupons were left immersed in the antimicrobial solutions for one hour at 22 °C. After treatments, the biocidal solutions were removed and the coupons were washed twice with 25 ml of D/E Neutralizing broth (Difco, Barcelona) followed by 25 ml BPW. Coupons were transferred to new Falcon test tubes containing 15 ml BPW. In order to resuspend the microbial cells attached to the biofilms, the coupons were rubbed on both sides with sterile cotton swabs followed by vortexing for 30 s. For each treatment replicate, the bacterial suspensions obtained from triplicate coupons were pooled together, centrifuged (3.500 × *g*, 30 min) and resuspended in a final volume of 5 ml SS. The resulting bacterial suspensions were serially diluted in SS and plated in triplicate on Trypticase soy agar (TSA, Scharlab, Barcelona). Viable cell counts obtained after 24 h incubation at 37 °C were used to calculate the average numbers of viable cells per ml.

### 2.4. DNA extraction, amplicon library preparation and sequencing

Aliquots (1.5 ml) of bacterial suspensions recovered from biofilms as described above were transferred to Eppendorf test tubes and centrifuged at 13.500 × *g* for 5 min to recover microbial cells. The pellets obtained from each sample were resuspended in 0.5 ml SS each. Then, Propidium Monoazide (PMA™, Biotium, UK) was added to block subsequent PCR amplification of the genetic material from dead cells as described by Elizaquivel, Sánchez, and Aznar (2012). DNA from PMA-treated cells was extracted by using a GenElute™ Bacterial Genomic DNA Kit (Sigma-Aldrich, Madrid), following instructions provided by the manufacturer. Briefly, cell pellets were treated with lysozyme solution (40 mg/ml) for 30 min at 37 °C and then incubated with the extraction kit lysis solution. DNA from the lysate was purified on the extraction kit purification columns and finally eluted with 100 μl of the Tris-EDTA buffer. DNA concentration and quality were measured with a NanoDrop spectrophotometer (Thermo Scientific, United Kingdom).

For pyrosequencing, V3–V5 region of the 16S rRNA gene was amplified using key-tagged bacterial primers prepared by Life-sequencing S.L. (Valencia, Spain) based on Sim et al. (2012). Polymerase chain reactions (PCR) were performed with 20 ng of community DNA, 200 μM of each of the four deoxynucleoside triphosphates, 400 nM of each primer, 2.5 U of FastStart HiFi Polymerase, and the appropriate buffer with $MgCl_2$ supplied by the manufacturer (Roche, Mannheim, Germany), 4% of 20 mg/ml bovine serum albumin (BSA) (Sigma, Dorset, United Kingdom), and 0.5 M Betaine (Sigma). Thermal cycling consisted of initial denaturation at 94 °C for 2 min followed by 30 cycles of denaturation at 94 °C for 20 s, annealing at 50 °C for 30 s, and extension at 72 °C for 5 min. To obtain sufficient material, PCR reactions were repeated in triplicate and pooled prior to purification by running the PCR amplicons on 1% (w/v) agarose gels. Amplicons were quantified using the PicoGreen assay (Quant-iT, PicoGreen DNA assay, Invitrogen) and combined in a single tube in equimolar concentrations. The pooled amplicon mixture was purified twice (AMPure XP kit, Agencourt, Takeley, United Kingdom) and the cleaned pool requantified with PicoGreen assay. Amplicons were submitted to the pyrosequencing services offered by Life Sequencing S.L. (Valencia, Spain) where emulsion-based clonal amplification (emPCR) was performed and subsequently, unidirectional pyrosequencing was carried out on a 454 Life Sciences GS FLX + instrument (Roche) following the Roche Amplicon Lib-L protocol.

### 2.5. Bioinformatic analysis

Bioinformatic analysis was carried out by Life Sequencing S.L. services. Raw reads were first filtered according to the 454 processing pipeline. Pyrosequencing reads were filtered with Q20 FASTX_tool_kit version 0.0.14, and reads were excluded from the analysis if they had an average quality score < Q20 and if there were ambiguous base calls (Ns). Reads were trimmed for adaptors and PCR primers, and only reads greater than 300 nts were retained for analysis. Chimera were eliminated using the Uchime algorithm under default mode (UCHIME version 4.2.40). The average final lengths of reads ranged from 549 to 562 nt. Sequences were compared with the National Center for Biotechnology Information (NCBI) 16 S rRNA database using the Basic Local Alignment Tool for Nucleotide sequences (BLASTN). Operational taxonomic units (OTUs) were defined by a 97% similarity. The read clusters were further assigned to taxonomies using the Ribosomal Database Project (RDP) classifier.

### 2.6. Statistical analysis

Viable cell counts were expressed as the average data from two replicates ± standard deviations. In order to determine if differences in viable counts between controls and treated samples were statistically significant, a paired *t*-test was performed at the 95% confidence interval. Pyrosequencing data were compared by Principal component analysis (PCA).

## 3. Results

### 3.1. Effect of treatments on biofilm viable cell counts

Enterocin AS-48 had only limited effect on total aerobic mesophilic counts recovered from biofilms (Table 1). The effect of treatments with BC was concentration-dependent. While BC solutions of 0.01 g/l had no effect on cell viability, statistically significant ($P < 0.05$) reductions of 1 and 4.1 log cycles were obtained for biocide concentrations of 0.1 and 1.0 g/l, respectively. The combination of BC and enterocin AS-48 did not reduce viable counts significantly ($P < 0.05$) for BC concentrations of 0.01 or 0.1 g/l compared to treatments with BC alone. However, for the highest BC concentration tested, viable counts were reduced below detectable levels in the combined treatment compared to 3.8 $\log_{10}$ CFU/ml in the single treatment (Table 1).

### 3.2. Effect of treatments on biofilm bacterial diversity

Total DNA was extracted from control biofilms and from biofilms treated with BC (at 0.1 g/l), enterocin AS-48, or plus enterocin AS-48 in order to study the bacterial biodiversity of the biofilms by pyrosequencing. Treatment with BC at 0.1 g/l was chosen since this biocide concentration significantly reduced viable cell counts while still leaving a sufficiently high number of residual viable cells as to do the microbial diversity study.

**Table 1**
Viable cell counts ($\text{Log}_{10}$ CFU/ml) from biofilms formed on stainless steel coupons and treated or not with enterocin AS-48, benzalkonium chloride (BC), or combinations of both.

| | Biocide concentration (g/l) | | | |
|---|---|---|---|---|
| | 0 | 0.01 | 0.1 | 1.0 |
| Control | 7.97 | | | |
| AS-48 | 7.87 | | | |
| BC | | 7.89 | 6.87 | 3.78 |
| BC + AS-48 | | 7.77 | 6.14 | <1.0 |

The microbiota recovered from control biofilms was composed mainly by *Proteobacteria* (99.90%). The most representative operational taxonomic units (OTUs) belonged to genera *Pseudomonas* (mainly *Pseudomonas entomophila*), followed by *Stenotrophomonas* (*Stenotrophomonas maltophilia*) (Table 2). The genera *Morganella*, *Achromobacter*, *Citrobacter*, *Cronobacter*, *Klebsiella* and *Delftia* had much lower relative abundances and were represented by a predominant species each. For *Klebsiella*, the predominant species found (*Klebsiella oxytoca*) had a relative abundance below 0.5% and was not included in the analysis. Treatment with enterocin AS-48 alone did not have a remarkable effect on the bacterial diversity of samples. The relative abundance of *Pseumonomas* (*P. entomophila*) was lower in the samples treated with enterocin AS-48, but the relative abundance of *S. maltophilia* was higher (Table 2). Treatment with BC reduced the relative abundance of *Pseudomonas* and other minor genera (*Citrobacter*, *Delftia*) and increased the relative abundance of *Stenotrophomonas* and *Morganella*. The same effect was observed for the combined treatment with BC plus enterocin (Table 2).

Principal component analysis of the data obtained at genus level revealed the strong association of *Pseudomonas* and *Stenotrophomonas* with the studied biofilms (Fig. 1). It also revealed high similarity between controls and treated samples. The similarity was highest for samples treated with AS-48 and BC plus AS-48 (Fig. 1). The calculated Pearson correlation coefficients between the different samples were always >0.95 with a $P < 0.05$.

## 4. Discussion

Biofilms are well-known reservoirs of food spoiling and human pathogenic bacteria. Previous studies show that sessile bacteria are far more resistant to disinfection than planktonic cells. This was corroborated in the present study using benzalkonium chloride (BC) as model biocide. The activity of BC on sessile bacteria from pure cultures (*B. cereus*, *L. monocytogenes*, *S. aureus*, *S. enterica*) was shown to be potentiated by enterocin AS-48 in previous studies (Caballero Gómez et al., 2012, Caballero Gómez, Grande et al., 2013a, Caballero Gómez, Abriouel et al., 2013b; Grande Burgos et al., 2012). However, there are no previous studies on its effects on biofilms formed from epiphytic microbiota. Results from the present work indicated a high disinfection resistance of the biofilms formed on stainless steel coupons by microbiota recovered from fresh produce (lettuce leaves, endives and cucumbers). Results also revealed that enterocin AS-48 could enhanced the antimicrobial activity of BC on the studied biofilms. However, this only occurred at high biocide concentrations. Cationic compounds, such as BC are thought to interact with negative charges in the bacterial cell wall and outer membrane (Gilber & Moore, 2005). They damage the cell wall and outer membrane of Gram-negative bacteria and promote their own uptake so that they can reach their target site(s) at the cell cytoplasmic membrane and within the cell cytoplasm (Maillard, 2002). A high concentration of BC would be expected to have a more pronounced chaotropic effect, especially on the outer membrane of Gram-negative bacteria, facilitating the diffusion of bacteriocin molecules to the bacterial cytoplasmic membrane, which is also the primary target for enterocin AS-48 (Gálvez, Maqueda, Martínez-Bueno, & Valdivia, 1991). Previous studies have shown that enterocin AS-48 is very active on gram positive bacteria, but it has very low or no activity on gram negative bacteria because of the outer membrane barrier (reviewed by Grande Burgos et al., 2014). Treatments disturbing the bacterial outer membrane (such as sublethal heat or chelators) have shown to enhance bacteriocin activity on gram negative bacteria (Abriouel, Valdivia, Gálvez, & Maqueda, 1998; Ananou, Gálvez, Martínez-

**Table 2**
Relative abundance of operational taxonomic units (OTUs) from control biofilm samples and from samples treated with benzalkonium chloride (BC), enterocin AS-48 or a combination of both.

| Genus | Species | Relative abundance (%)[a] | | | |
|---|---|---|---|---|---|
| | | Control | AS-48 | BC | BC + AS-48 |
| *Pseudomonas* | | 70.99 | 63.69 | 55.70 | 60.97 |
| | *Pseudomonas entomophila* | 64.78 | 55.09 | 54.47 | 59.62 |
| | *Pseudomonas plecoglossicida* | 4.77 | 7.34 | 0.28 | 0.77 |
| | *Pseudomonas hibiscicola* | 1.70 | 1.58 | 0.07 | 0.05 |
| | *Pseudomonas aeruginosa* | 0.055 | 0.07 | 0.83 | 0.32 |
| | *Pseudomonas monteilii* | 0.70 | 0.46 | 0.10 | 0.07 |
| | *Pseudomonas fulva* | 0.48 | 0.51 | 0.02 | 0.14 |
| *Stenotrophomonas* | | 25.38 | 31.97 | 38.31 | 31.70 |
| | *Stenotrophomonas maltophilia* | 23.68 | 30.39 | 38.23 | 31.65 |
| *Morganella* | | 0.22 | 0.11 | 1.09 | 1.35 |
| | *Morganella morganii* | 0.20 | 0.09 | 1.02 | 1.29 |
| *Achromobacter* | | 0.66 | 0.53 | 1.03 | 1.65 |
| | *Achromobacter insolitus* | 0.50 | 0.29 | 0.65 | 1.40 |
| *Citrobacter* | | 0.94 | 0.61 | 0.39 | 0.65 |
| | *Citrobacter freundii* | 0.55 | 0.35 | 0.15 | 0.36 |
| *Cronobacter* | | 0.35 | 0.40 | 0.54 | 0.84 |
| | *Cronobacter dublinensis* | 0.28 | 0.37 | 0.50 | 0.65 |
| *Klebsiella* | | 0.09 | 0.40 | 0.53 | 0.88 |
| *Delftia* | | 0.53 | 0.51 | 0.10 | 0.02 |
| | *Delftia tsuruhatensis* | 0.53 | 0.50 | 0.10 | 0.02 |

[a] Only ranks with a relative abundance of at least 0.5% in at least one of the samples are represented.

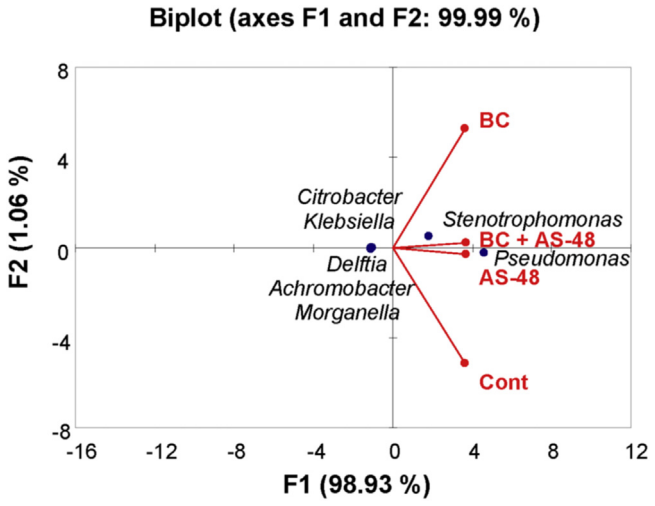


**Fig. 1.** Biplot principal component analysis of sample variables (treatments) and scores (genus with relative abundance ≥ 0.5%).

Bueno, Maqueda, & Valdivia, 2005; Cobo Molinos et al., 2009). Therefore, it was important to study the composition of the studied biofilms in order to determine if they were formed predominantly by gram negative bacteria.

High-throughput sequencing results revealed dual-species biofilms dominated by *Proteobacteria* of the genera *Pseudomonas* and *Stenotrophomonas* with other minor genera/species associated. These results would explain the lack of activity of enterocin AS-48 when tested singly or in combination with low concentrations of BC. The composition of biofilms would be expected to be influenced by environmental factors (such as pH, temperature, nutrients and oxygen availability) and by the complex interactions among the different microbial populations in the biofilm. *Pseudomonas* and *Stenotrophomonas* are known to produce antimicrobial substances such as pyocins, lectin-like bacteriocins, modified microcins, or maltocins (Dong, Zhu, Chen, Ye, & Huang, 2015; Ghequire & De Mot, 2014; Michel-Briand & Baysse, 2002). Production of antimicrobial substances could displace competitors (either of the same species or even of unrelated bacterial genera or fungi) and at the same time enhance biofilm formation (Oliveira et al., 2015). Both bacterial groups are also known to release membrane vesicles, which may be important for biofilm formation (Ferrer-Navarro et al., 2016; Tashiro, Uchiyama, & Nomura, 2012). In particular, a process called explosive lysis (by which a small fraction of the bacterial population undergo cell lysis and release a variety of cellular components including cytosolic proteins, DNA and membrane vesicles) has been shown to enhance biofilm formation by *Pseudomonas* (Turnbull et al., 2016).

Another relevant factor for biofilm formation would be the microbial composition of the source of contamination. In the present study, the presence of *Pseudomonas* in the preparations used to inoculate the coupons would be expected, since previous studies have shown the relevance of gram-negative bacteria and in particular *Pseudomonas* sp. among the microbiota of lettuce (King, Magnuson, Török, & Goodman, 1991; Lee et al., 2013; Rastogi et al., 2012). *Pseudomonas entomophila, Raphanus sativus, Lactuca sativa, Pseudomonas*, *Stenotrophomonas*. It has been shown that broad-leaved endive harbors biofilms containing fluorescent pseudomonads (Boureau et al., 2004). *S. maltophilia* is capable of forming biofilms on a number of materials such as polystyrene, popypropilene, borosilicate or stainless steel (Leriche, Sibille, & Carpentier, 2000; Zgair & Chhibber, 2013). In one study, *S. maltophilia* was cultured from 14 (78%) of 18 washed and ready to eat salads investigated (Qureshi, Mooney, Denton, & Kerr, 2005). Those results were reported to be a matter of concern since *S. maltophilia* has emerged as an important nosocomial pathogen, especially in debilitated and immunocompromised persons (Denton & Kerr, 1998). Biofilm formation by spoiling and potentially pathogenic bacteria of the genera *Pseudomonas* and *Stenotrophomonas* on surfaces of vegetable food processing facilities should be further investigated.

This is the first report showing that combined treatments of enterocin AS-48 and BC reduce the total viable cell counts in biofilms formed by microbiota from fresh produce, composed mainly by *Pseudomonas* (predominantly *P. entomophila*) and *S. maltophilia* according to pyrosequencing data. Further studies on biofilms formed by *Pseudomonas* and *Stenotrophomonas* strains isolated

from fresh produce need to be carried out in order to determine possible strain and/or species differences in sensitivity to the combined treatment. Inclusion of other antimicrobials such as essential oils or phenolic compounds in the combined treatments should also be tested in order to improve the efficacy of treatments while reducing the final concentration of BC.

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## References

Abriouel, H., Lucas, R., Ben Omar, N., Valdivia, E., & Gálvez, A. (2010). Potential applications of the cyclic peptide enterocin AS-48 in the preservation of vegetable foods and beverages. *Probiotics and Antimicrobial Proteins, 2*, 77–89.

Abriouel, H., Valdivia, E., Gálvez, A., & Maqueda, M. (1998). Response of *Salmonella choleraesuis* LT2 spheroplasts and permeabilized cells to the action of the bacteriocin AS-48. *Applied and Environmental Microbiology, 64*, 4623–4626.

Abriouel, H., Valdivia, E., Martínez-Bueno, M., Maqueda, M., & Gálvez, A. (2003). Method for semi-preparative-scale production and recovery of enterocin AS-48 derived from *Enterococcus faecalis* subsp. *liquefaciens* A-48-32. *Journal of Microbiological Methods, 55*, 599–605.

Ananou, S., Gálvez, A., Martínez-Bueno, M., Maqueda, M., & Valdivia, E. (2005). Synergistic effect of enterocin AS-48 in combination with outer membrane permeabilizing treatments against *Escherichia coli* O157:H7. *Journal of Applied Microbiology, 99*, 1364–1372.

Boureau, T., Jacques, M. A., Berruyer, R., Dessaux, Y., Dominguez, H., & Morris, C. E. (2004). Comparison of the phenotypes and genotypes of biofilm and solitary epiphytic bacterial populations on broad-leaved endive. *Microbial Ecology, 47*(1), 87–95.

Branda, S. S., Vik, A., Friedman, L., & Kolter, R. (2005). Biofilms: The matrix revisited. *Trends in Microbiology, 13*, 20–26.

Caballero Gómez, N., Abriouel, H., Grande, M. A., Pérez Pulido, R., & Gálvez, A. (2012). Effect of enterocin AS-48 in combination with biocides on planktonic and sessile *Listeria monocytogenes*. *Food Microbiology, 30*, 51–58.

Caballero Gómez, N., Abriouel, H., Grande, M. J., Pérez Pulido, R., & Gálvez, A. (2013b). Combined treatments of enterocin AS-48 with biocides to improve the inactivation of methicillin-sensitive and methicillin-resistant *Staphylococcus aureus* planktonic and sessile cells. *International Journal of Food Microbiology, 163*, 96–100.

Caballero Gómez, N., Grande, M. J., Pérez Pulido, R., Abriouel, H., & Gálvez, A. (2013a). Effect of enterocin AS-48 singly or in combination with biocides on planktonic and sessile *Bacillus cereus*. *Food Control, 34*, 743–751.

Cobo Molinos, A., Lucas, R., Abriouel, H., Ben Omar, N., Valdivia, E., & Gálvez, A. (2009). Inhibition of *Salmonella enterica* cells in deli-type salad by enterocin AS-48 in combination with other antimicrobials. *Probiotics and Antimicrobial Proteins, 1*, 85–90.

Denton, M., & Kerr, K. G. (1998). Microbiological and clinical aspects of infections associated with *Stenotrophomonas maltophilia*. *Clinical Microbiology Reviews, 11*, 57–80.

Dong, H., Zhu, C., Chen, J., Ye, X., & Huang, Y. P. (2015). Antibacterial activity of *Stenotrophomonas maltophilia* endolysin P28 against both gram-positive and gram-negative bacteria. *Frontiers in Microbiology, 6*, 1299.

Elizaquivel, P., Sánchez, G., & Aznar, R. (2012). Quantitative detection of viable foodborne *Escherichia coli* O157:H7, *Listeria monocytogenes* and *Salmonella* in fresh-cut vegetables combining propidium monoazide and real-time PCR. *Food Control, 25*, 704–708.

Ferrer-Navarro, M., Torrent, G., Mongiardini, E., Conchillo-Solé, O., Gibert, I., & Daura, X. (2016). Proteomic analysis of outer membrane proteins and vesicles of a clinical isolate and a collection strain of *Stenotrophomonas maltophilia*. *Journal of Proteomics, 142*, 122–129.

Gálvez, A., Maqueda, M., Martínez-Bueno, M., & Valdivia, E. (1991). Permeation of bacterial cells, permeation of cytoplasmic and artificial membrane vesicles, and channel formation on bilayers by peptide antibiotic AS-48. *Journal of Bacteriology, 173*, 886–892.

Ghequire, M. G., & De Mot, R. (2014). Ribosomal encoded antibacterial proteins and peptides from *Pseudomonas*. *FEMS Microbiology Reviews, 38*(4), 523–568.

Gilber, P., & Moore, L. E. (2005). Cationic antiseptics: Diversity of action under a common epithet. *Journal of Applied Microbiology, 99*, 703–715.

Gilbert, P., Das, J. R., Jones, M. V., & Allison, D. G. (2001). Assessment of resistance towards biocides following the attachment of microorganisms to, and growth on, surfaces. *Journal of Applied Microbiology, 91*, 248–254.

Gilbert, P., McBain, A. J., & Rickard, A. H. (2003). Formation of microbial biofilm in hygienic situations: A problem of control. *International Biodeterioration and Biodegradation, 51*, 245–248.

Grande Burgos, M. J., Lucas López, R., López Aguayo, M. C., Pérez Pulido, R., & Gálvez, A. (2012). Inhibition of planktonic and sessile *Salmonella enterica* cells by combinations of enterocin AS-48, polymyxin B and biocides. *Food Control, 30*, 214–221.

Grande Burgos, M. J., Pérez Pulido, R., López Aguayo, M. C., Gálvez, A., & Lucas, R. (2014). The cyclic antibacterial peptide enterocin AS-48: Isolation, mode of action, and possible food application. *International Journal of Molecular Sciences, 15*, 22706–22727.

Hall-Stoodley, L., Costerton, J. W., & Stoodley, P. (2004). Bacterial biofilms: From the natural environment to infectious diseases. *Nature Reviews Microbiology, 2*, 95–108.

Jefferson, K. K. (2004). What drives bacteria to produce a biofilm? *FEMS Microbiology Letters, 236*, 163–173.

Jessen, B., & Lammert, L. (2003). Biofilm and disinfection in meat processing plants. *International Biodeterioration and Biodegradation, 51*, 265–269.

King, A. D., Magnuson, J. A., Török, T., & Goodman, N. (1991). Microbial flora and storage quality of partially processed lettuce. *Journal of Food Science, 56*, 459–461.

Krysinski, E. P., Brown, L. J., & Marchisello, T. J. (1992). Effect of cleaners and sanitizers on *Listeria monocytogenes* attached to product contact surfaces. *Journal of Food Protection, 55*, 246–251.

Kuda, T., Yano, T., & Kuda, M. T. (2008). Resistances to benzalkonium chloride of bacteria dried with food elements on stainless steel surface. *LWT - Food Science and Technology, 41*, 988–993.

Lee, D. H., Kim, J. B., Kim, M., Roh, E., Jung, K., Choi, M., et al. (2013). Microbiota on spoiled vegetables and their characterization. *Journal of Food Protection, 76*, 1350–1358.

Leriche, V., Sibille, P., & Carpentier, B. (2000). Use of an enzyme-linked lectino-sorbent assay to monitor the shift in polysaccharide composition in bacterial biofilms. *Applied and Environmental Microbiology, 66*, 1851–1856.

Maillard, J. Y. (2002). Bacterial target sites for biocide action. *Journal of Applied Microbiology, 92*, 16–27.

Maqueda, M., Gálvez, A., Martínez-Bueno, M., Sánchez-Barrena, J., González, C., Albert, A., et al. (2004). Peptide AS-48: Prototype of a new class of cyclic bacteriocins. *Current Protein & Peptide Science, 5*, 399–416.

Michel-Briand, Y., & Baysse, C. (2002). The pyocins of *Pseudomonas aeruginosa*. *Biochimie, 84*(5–6), 499–510.

Morton, L. H. G., Greenway, D. L. A., Gaylarde, C. C., & Surman, S. B. (1998). Consideration of some implications of the resistance of biofilms to biocides. *International Biodeterioration and Biodegradation, 41*, 247–259.

Oliveira, N. M., Martinez-Garcia, E., Xavier, J., Durham, W. M., Kolter, R., Kim, W., et al. (2015). Biofilm Formation as a response to ecological competition. *PLOS Biology, 13*(7), e1002191.

Qureshi, A., Mooney, L., Denton, M., & Kerr, K. G. (2005). *Stenotrophomonas maltophilia* in salad. *Emerging Infectious Diseases Journal, 11*(7), 1157–1158.

Rastogi, G., Sbodio, A., Tech, J. J., Suslow, T. V., Coaker, G. L., & Leveau, J. H. J. (2012). Leaf microbiota in an agroecosystem: Spatiotemporal variation in bacterial community composition on field-grown lettuce. *The ISME Journal, 6*, 1812–1822.

Sim, K., Cox, M. J., Wopereis, H., Martin, R., Knol, J., Li, M. S., et al. (2012). Improved detection of bifidobacteria with optimised 16S rRNA gene based pyrosequencing. *PLoS One, 7*(3), e32543.

Tashiro, Y., Uchiyama, H., & Nomura, N. (2012). Multifunctional membrane vesicles in *Pseudomonas aeruginosa*. *Environmental Microbiology, 14*(6), 1349–1362.

Turnbull, L., Toyofuku, M., Hynen, A. L., Kurosawa, M., Pessi, G., Petty, N. K., et al. (2016). Explosive cell lysis as a mechanism for the biogenesis of bacterial membrane vesicles and biofilms. *Nature Communications, 7*, 11220.

Ueda, S., & Kuwabara, Y. (2007). Susceptibility of biofilm *Escherichia coli, Salmonella* Enteritidis and *Staphylococcus aureus* to detergents and sanitizers. *Biocontrol Science, 12*, 149–153.

Van Houdt, R., & Michiels, C. W. (2010). Biofilm formation and the food industry, a focus on the bacterial outer surface. *Journal of Applied Microbiology, 109*, 1117–1131.

Yaron, S., & Römling, U. (2014). Biofilm formation by enteric pathogens and its role in plant colonization and persistence. *Microbial Biotechnology, 7*, 496–516.

Zgair, A. K., & Chhibber, S. (2013). *Stenotrophomonas maltophilia* flagellin is involved in bacterial adhesion and biofilm formation. *Microbiology, 82*, 647–651.