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# Characterization of the microflora from a minimally processed vegetables plant and development of innovative strategies for microbial growth control

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Dissertation submitted to obtain the degree of Doctor in Chemical and Biological Engineering

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# Characterization of the microflora from a minimally processed vegetables plant and development of innovative strategies for microbial growth control

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## THESIS OUTPUTS

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Meireles, A., Machado, I., Fulgêncio, R., Mergulhão, F., Melo, L., Simões, M. (2015). Efficacy of antimicrobial combinations to reduce the use of sodium hypochlorite in the control of planktonic and sessile *Escherichia coli*. Biochemical Engineering Journal. 104. 115-122.

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## ETHICS STATEMENT

All the experiments and data analysis were performed by the PhD student.

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The student — The Supervisor

Ana Meireles — Manuel Simões

## ABSTRACT

Microbial contamination is an unavoidable problem in industrial processes and the foodborne illness outbreaks linked to minimally processed vegetables (MPV) are becoming more frequent and widespread. Sodium hypochlorite (SH) is the most common biocide used for industrial disinfection and has been identified as a concern mainly due to the potential production of toxic by-products, such as chloroform and other trihalomethanes, chloramines and haloacetic acids, when in contact with organic matter. The search for alternative methods of disinfection is therefore a current and on-going challenge in food industry. Consequently, in this work the action of chlorine dioxide (CD), peracetic acid (PA), hydrogen peroxide (HP), copper sulfate (CS), vanillin (VN) and sodium bicarbonate (SB), alone and combined with SH, were evaluated on the control of planktonic and sessile *Escherichia coli* CECT 434. The most effective disinfectants were PA (460 ppm) and CD (200 ppm) and the best combination with SH (225 ppm) was obtained with PA (150 ppm).

As chlorine is extremely efficient, the efficacy of three alternative chlorine-based biocides (neutral electrolyzed oxidizing water (NEOW), CD and sodium dichloroisocyanurate (NaDCC)), was also assessed and compared with SH. These tests were performed against *E. coli* in both planktonic and sessile states. The planktonic tests revealed that SH had the fastest antimicrobial action, NaDCC exhibited the highest antimicrobial rate and NEOW had the highest antimicrobial action. In biofilm control, NEOW and CD were more efficient, followed by NaDCC and SH. In terms of stability, NEOW had the longest decay time for chlorine loss (70 days at 5 ºC) and the lowest chlorine loss rate (0.013 $ppm.min^{-1}$ at 5 ºC). The overall results propose that NEOW can be a good alternative to SH due to its higher antimicrobial/antibiofilm effects and stability.

The knowledge on the microorganisms present in an industrial process is crucial to delineate the best strategy for their effective control. Therefore, the resident heterotrophic bacteria present in a MPV plant was isolated, identified and characterized (in terms of virulence factors production (proteases, gelatinases and siderophores), quorum sensing

inhibition, biofilm formation, antibiotic resistance, aggregation potential, stomach environment survival, and susceptibility to biocides (SH and PA) in planktonic and sessile states). Twenty different bacteria were identified: *Arthrobacter* sp., *Bacillus* sp. *Bacillus aryabhattai*, *Exiguobacterium* sp., *Microbacterium maritypicum*, *Micrococcus luteus*, *Rathayibacter caricis*, *Rhodococcus erythropolis*, *Streptococcus salivarius*, *Chryseobacterium indoltheticum*, *Enterobacteriaceae bacterium*, *Erwinia* sp., *Pseudomonas* sp., *Pseudomonas poae*, *Pseudomonas oryzihabitans*, *Pseudomonas putida*, *Rahnella aquatilis*, *Sphingobacterium faecium*, *Stenotrophomonas maltophilia* and *Xanthomonas campestris*. The conveyor belt in the high care area was found to be a significant source of contamination. Most of the isolates were capable of producing virulence related molecules. *Pseudomonas* was the genera with the highest biofilm formation ability, being the predominant microflora along the process chain. It was found that Gram-negative bacteria were more resistant to the selected antibiotics than Gram-positive. Also, *E. bacterium*, *Erwinia* sp., *P. oryzihabitans*, *P. putida*, *R. aquatilis* and *S. maltophilia* were considered multidrug resistant. All bacteria were eliminated in the simulated gastric environment. In addition, *R. erythropolis* was able to autoaggregate and no evidence of coaggregation was found. The biofilm experiments demonstrated that PA action was lower than that of SH in killing the isolates. Additionally, neither of the disinfectants was capable of removing the biofilm cells. Furthermore, biofilm regrowth was observed after disinfectant exposure, proposing the modest action of the selected disinfectants in biofilm control and the need for alternative and improved strategies for disinfection. Even if no significant pathogen was isolated, the results clearly propose that improvements in decontamination during processing are required to effectively control microbial presence in the final product.

The tests with the food isolates proved that *E. coli* had a different behavior concerning disinfection. To understand such differences, a food isolate (*P. oryzihabitans*) was compared with *E. coli*. It was possible to verify that *P. oryzihabitans* produced much more siderophores, was more tolerant to SH and to the tested antibiotics and was less susceptible to PA. Therefore, effective disinfection strategies should target the contaminating microorganisms and the reference strains should be carefully selected when validating methods and processes. Additionally, in the laboratory tests the media used do not resemble the actual MPV. Thus, a new medium that mimics the lettuce chemical composition was developed, tested and compared with a commonly used

medium in antimicrobial tests (Mueller–Hinton broth (MHB)). *E. coli* formed more biofilm in the artificial lettuce medium and the application of SH and PA caused lower log CFU (colony forming units) reductions when the cells were in the new medium. The results obtained demonstrated that using a medium similar to lettuce, in chemical composition, for microbiological tests, led to different results, particularly the increase of antimicrobial tolerance.

**Keywords:** Alternative; Biofilm control; Food safety; Efficacy; Disinfection; Neutral electrolyzed oxidizing water; Peracetic acid; Sodium hypochlorite, Stability.

## RESUMO

A contaminação microbiana é um problema inevitável em processos industriais e os surtos de doenças transmitidas pelos vegetais minimamente processados estão a tornar-se frequentes e generalizados. O hipoclorito de sódio é o biocida mais utilizado para desinfeção industrial e tem sido considerado problemático devido à produção de subprodutos tóxicos, como clorofórmio e outros trialometanos, cloraminas e ácidos haloacéticos, quando entra em contacto com a matéria orgânica. Por este motivo, a pesquisa por métodos alternativos de desinfeção é um desafio atual na indústria alimentar. Consequentemente, neste trabalho dióxido de cloro, ácido peracético, peróxido de hidrogénio, sulfato de cobre, vanilina e bicarbonato de sódio foram avaliados individualmente e em combinação com o hipoclorito de sódio no controlo de *Escherichia coli* CECT 434 no estado planctónico e séssil. Os desinfetantes mais eficazes foram o ácido peracético (460 ppm) e o dióxido de cloro (200 ppm) e a melhor combinação com o hipoclorito de sódio (225 ppm) foi obtida com o ácido peracético (150 ppm).

Como o cloro é extremamente eficiente, a eficácia de três biocidas com cloro (água neutra eletrolisada e oxidante, dióxido de cloro e dicloroisocianurato de sódio) foram avaliados e comparados com o hipoclorito de sódio. Estes testes foram realizados com *E. coli* no estado planctónico e séssil. Os testes planctónicos revelaram que o hipoclorito de sódio tinha a ação antimicrobiana mais rápida, o dicloroisocianurato de sódio exibiu a taxa antimicrobiana mais elevada e a água neutra eletrolisada e oxidante teve a ação antimicrobiana mais elevada. No controlo de biofilme a água neutra eletrolisada e oxidante e o dióxido de cloro foram os mais eficientes, seguidos pelo dicloroisocianurato de sódio e pelo hipoclorito de sódio. Relativamente à estabilidade, a água neutra eletrolisada e oxidante teve o tempo de decaimento mais longo para a perda de cloro (70 dias a 5 ºC) e a taxa de perda de cloro mais baixa (0.013 ppm.min$^{-1}$ a 5 ºC). Os resultados propõem que a água neutra eletrolisada e oxidante pode ser uma boa alternativa ao hipoclorito de sódio devido aos seus elevados efeitos antimicrobianos/antibiofilme e estabilidade.

O conhecimento dos microrganismos presentes num processo industrial é crucial para delinear a melhor estratégia para o seu controlo efetivo. Desta forma, as bactérias heterotróficas presentes na indústria de vegetais minimamente processados foram isoladas, identificadas e caracterizadas (produção de factores de virulência (proteases, gelatinases e sideróforos), inibição de *quorum sensing*, formação de biofilme, resistência a antibióticos, agregação, sobrevivência num ambiente estomacal, suscetibilidade aos biocidas (hipoclorito de sódio e ácido peracético) para células no estado planctónico e séssil). Foram identificadas vinte bactérias diferentes: *Arthrobacter* sp., *Bacillus* sp. *Bacillus aryabhattai*, *Exiguobacterium* sp., *Microbacterium maritypicum*, *Micrococcus luteus*, *Rathayibacter caricis*, *Rhodococcus erythropolis*, *Streptococcus salivarius*, *Chryseobacterium indoltheticum*, *Enterobacteriaceae bacterium*, *Erwinia* sp., *Pseudomonas* sp., *Pseudomonas poae*, *Pseudomonas oryzihabitans*, *Pseudomonas putida*, *Rahnella aquatilis*, *Sphingobacterium faecium*, *Stenotrophomonas maltophilia* e *Xanthomonas campestris*. O tapete transportador na área de cuidado elevado foi considerado como uma fonte de contaminação significativa. A maior parte dos isolados foi capaz de produzir moléculas de virulência. Microrganismos do género *Pseudomonas* foram os microrganismos capazes de formar mais biofilme sendo ainda as bactérias predominantes ao longo do processo. As bactérias Gram-negativas foram consideradas mais resistentes aos antibióticos selecionados do que as bactérias Gram-positivas. As bactérias *E. bacterium*, *Erwinia* sp., *P. oryzihabitans*, *P. putida*, *R. aquatilis* e *S. maltophilia* foram consideradas multirresistentes. Todas as bactérias foram eliminadas no ambiente gástrico simulado. Adicionalmente, *R. erythropolis* foi capaz de se autoagregar mas não foi detetada nenhuma evidência de coagregação. As experiências em biofilme demonstraram que a ação do ácido peracético foi inferior à ação do hipoclorito de sódio na eliminação dos isolados. Nenhum dos desinfetantes foi capaz de remover as células do biofilme. Adicionalmente, o recrescimento do biofilme foi observado após a exposição aos desinfetantes, propondo a ação modesta dos desinfetantes selecionados para o controlo de biofilme e ainda a necessidade de estratégias alternativas e melhores para a desinfeção. Mesmo que nenhum patogénico tenha sido isolado, os resultados propõem que devem ser efetuados melhoramentos na descontaminação durante o processamento para controlar efetivamente a presença microbiana no produto final.

Os testes com os isolados provaram que a *E. coli* tem um comportamento diferente no que concerne a desinfeção. De forma a entender essas diferenças, um isolado alimentar

(*P. oryzihabitans*) foi comparado com a *E. coli*. Foi possível verificar que a *P. oryzihabitans* produziu sideróforos numa quantidade superior, foi mais tolerante ao hipoclorito de sódio e aos antibióticos testados e foi menos suscetível ao ácido peracético. Deste modo, as estratégias de desinfeção devem ser dirigidas aos microrganismos contaminantes e as estirpes de referência devem ser selecionadas cuidadosamente quando se valida métodos e processos. Adicionalmente, nos testes laboratoriais o meio usado não se assemelha aos vegetais minimamente processados. Assim, um novo meio que mimetiza a composição química das alfaces foi testado e comparado com um meio comummente utilizado nos testes antimicrobianos (meio Mueller–Hinton). A *E. coli* formou mais biofilme no meio artificial de alface e a aplicação de hipoclorito de sódio e ácido peracético causaram reduções log de unidades formadoras de colónias mais baixas no novo meio. Os resultados obtidos demonstraram que utilizar um meio similar à alface, em composição química, para testes antimicrobianos, originou resultados diferentes, particularmente o aumento da tolerância antimicrobiana.

**Palavras-chave:** Ácido peracético; Água neutra eletrolisada e oxidante; Alternativas; Controlo de biofilme; Desinfeção; Eficácia; Estabilidade; Hipoclorito de sódio; Segurança alimentar.

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### Abbreviations

| | |
|---|---|
| AcEOW | Acid electrolyzed oxidizing water |
| AHL | *N*-acyl homoserine lactones |
| AI | Autoinducers |
| AlEOW | Alkaline electrolyzed oxidizing water |
| AMP | Ampicillin |
| BOD | Biochemical oxygen demand |
| BPW | Buffered peptone water |
| CAS | Chrome azurol S |
| CD | Chlorine dioxide |
| CFU | Colony forming units |
| CIP | Ciprofloxacin |
| COD | Chemical oxygen demand |
| CS | Copper sulfate |
| CV | Crystal violet |
| DCFH-DA | Dichloro-dihydro-fluorescein diacetate |
| DMSO | Dimethyl sulfoxide |
| EOW | Electrolyzed oxidizing water |
| EPS | Extracellular polymeric substances |
| FC | Free chlorine |
| FDA | Food and Drug Administration |

| | |
|---|---|
| GRAS | Generally recognized as safe |
| HOCl | Hypochlorous acid |
| HP | Hydrogen peroxide |
| HSL | Homoserine lactone |
| I | Intermediate |
| LAB | Lactic acid bacteria |
| LB | Luria-Bertani |
| LBB | Luria-Bertani broth |
| LEV | Levofloxacin |
| LTB | Lettuce broth |
| MBC | Minimal bactericidal concentration |
| MF | Microfiltration |
| MHA | Mueller–Hinton agar |
| MHB | Mueller–Hinton broth |
| MIC | Minimum inhibitory concentration |
| MPV | Minimally processed vegetables |
| MRS | Man, Rogosa and Sharpe broth |
| MWCO | Molecular weight cut off |
| NaDCC | Sodium dichloroisocyanurate |
| NEOW | Neutral electrolyzed oxidizing water |
| NF | Nanofiltration |
| O.D. | Optical density |
| $O.D._C$ | Optical density of the cut-off value |
| ORP | Oxidation reduction potential |
| PA | Peracetic acid |
| PCA | Plate count agar |

| | |
|---|---|
| PPO | Polyphenol oxidase |
| PS | Polystyrene |
| QACs | Quaternary ammonium compounds |
| QS | Quorum sensing |
| QSI | Quorum sensing inhibition |
| R | Resistant |
| RG | Regrowth |
| RMSE | Root mean square error |
| RNA | Ribonucleic acid |
| RO | Reverse osmosis |
| ROS | Reactive oxygen species |
| RTE | Ready-to-eat |
| S | Susceptible |
| SB | Sodium bicarbonate |
| SH | Sodium hypochlorite |
| SS | Stainless steel |
| TET | Tetracycline |
| TSA | Tryptic soy agar |
| TSB | Tryptic soy broth |
| UF | Ultrafiltration |
| US | Ultrasounds |
| UV | Ultraviolet |
| VN | Vanillin |

**Symbols**

| | |
|---|---|
| μ | Growth rate ($h^{-1}$) |

| | |
|---|---|
| $CFU(t_m)$ | CFU at a determined time $t_m$ (min) |
| $CFU_0$ | Initial value of CFU |
| $CFU_f$ | Final value of CFU |
| $FC(t_d)$ | FC at a determined time $t_d$ (days) |
| $FC_0$ | Initial value of the FC |
| $FC_f$ | Final value of the FC |
| i | End of the exponential phase |
| $k_{CFU}$ | Maximum antimicrobial rate ($min^{-1}$) |
| $k_{FC}$ | Maximum chlorine loss rate ($ppm.days^{-1}$) |
| LR | Log CFU reduction index |
| $LR_c$ | Log CFU reduction of the compound in the combination |
| $LR_i$ | Log CFU reduction of the compound used individually |
| $MIC_c$ | MIC of the compound in the combination |
| $MIC_i$ | MIC of the compound used individually |
| $t_d$ | Time (days) |
| T | Time (h) |
| $T_d$ | Generation or doubling time (h) |
| $t_m$ | Time (min) |
| $\lambda_{CFU}$ | Time for antimicrobial action (min) |
| $\lambda_{FC}$ | Time for chlorine loss (days) |
| 0 | Beginning of the exponential phase |

# 1

## INTRODUCTION

In this section the background and motivation will be presented. Additionally the main objectives will be outlined and described.

## 1.1. Background and motivation

Nowadays, consumers are more conscious of the importance of a healthy life and with what they eat (Seiber, 2012). For this reason, the consumption of fresh produce has increased over the years, as they are a natural source of nutrients, are easy to use and have already been washed, thus reducing water consumption by the consumer (Seiber, 2012). Minimally processed vegetables (MPV) or ready-to-eat (RTE) vegetables are defined as fresh produce that are pre-washed and cut, to a minimal level, and packaged (Ragaert et al., 2004). This kind of product does not have a long shelf-life, since the current decontamination procedures have a reduced efficiency and the cutting processes increase the availability of nutrients for the microorganisms (Siroli et al., 2015). However, with the increasing consumption of MPV the illness outbreaks associated to human pathogens have also increased (Foong-Cunningham et al., 2012, Olaimat and Holley, 2012). The main microorganisms responsible for foodborne illness outbreaks associated with fresh produce are *E. coli* O157:H7 and *Salmonella* spp. (Warriner et al., 2009). Nevertheless, human infections can be caused by other foodborne pathogens, e.g. *Listeria monocytogenes*, *Clostridium botulinum*, *Bacillus cereus* (Warriner et al., 2009, Seiber, 2012), norovirus (Van Boxstael et al., 2013, Mritunjay and Kumar, 2015) and protozoa (*Cryptosporidium parvum*) (Yaron and Romling, 2014). These outbreaks are linked to major economic losses. Only in the USA, contaminated fresh produce is responsible for 20 million illnesses representing 38.6 $ billion every year (Olaimat and Holley, 2012, Jeddi et al., 2014).

Contamination of fresh produce can occur through the water, air, soil, fertilizers, insect vectors, equipment or even through the improper handling by the workers (Martinez-Vaz et al., 2014). For instance, microbial adhesion on food-contact surfaces (i.e. equipment including conveyor belts and containers used along the food chain - in harvesting, post-harvesting and packaging (Food and Drug Administration, 1998)) can ultimately lead to the formation of biofilms (Yaron and Romling, 2014) and the subsequent produce contamination. Biofilms are sessile communities of microorganisms that initially attach to a wet solid surface, and subsequently grow producing extracellular polymeric

substances (EPS) that keep the cells strongly together and also protect them from external stress conditions (Kumar and Anand, 1998). Biofilms have a negative impact as they can be formed on the produce and on the food-contact surfaces impairing surface sanitation and causing produce contamination (Kumar and Anand, 1998, Martinez-Vaz et al., 2014). More importantly, microbial contamination can also lead to the internalization of pathogens into the produce. For instance, both *E. coli* and *Salmonella* Typhimurium are capable of penetrating the leaves of iceberg lettuce (Golberg et al., 2011). Seo and Frank (1999) demonstrated that *E. coli* O157:H7 can penetrate 20-100 µm below the surface of lettuce leaves. In another work, Kroupitski et al. (2009) showed that through chemotaxis processes and flagellar motility, *Salmonella* spp. can penetrate lettuce leaves. The internalization can occur in the stomata, vasculature, cut edges, intercellular tissues, etc. (Erickson, 2012). Consequently, the elimination of such pathogens already internalized in the produce is rather impossible, making the subsequent minimal processing totally ineffective to assure produce safety (Erickson, 2012, Ge et al., 2013). Furthermore, MPV are also susceptible to colonisation by spoilage microorganisms (Olaimat and Holley, 2012). Spoilage is associated with any food modification, which in turn causes changes in the texture, colour, odour and flavour, affecting their organoleptic properties. These modifications can be caused by physical damages and chemical or microbiological reactions (Gram et al., 2002, Degl'Innocenti et al., 2005, Madigan et al., 2009). All microorganisms that are present in the food and persist after decontamination can be considered spoiling, since they were able to resist and can cause changes in the organoleptic properties of the product (Gram et al., 2002).

Produce outbreaks and spoilage have considerable economic consequences, since the company has to afford the associated medical costs; has to deal with litigation lawsuits; has to dispose of the affected produce; and the company's reputation is also harmed (Sapers and Doyle, 2009, Foong-Cunningham et al., 2012). However, as the disinfection and decontamination of the produce is minimal, pathogenic and spoilage microorganisms are not always eliminated with chemical products (Foong-Cunningham et al., 2012, Olaimat and Holley, 2012). In the MPV industry, decontamination of the produce and sanitization of the food-contact surfaces can be achieved by applying the adequate decontaminants/disinfectants, typically sodium hypochlorite (SH). SH is effective against a wide range of microorganisms, has a low price and can be easily applied (Ramos et al., 2013). However, its action is not suitably effective in pathogens elimination (Yaron and

Romling, 2014) and it interacts strongly with organic matter originating carcinogenic and mutagenic organochlorinated by-products (Bull et al., 2011). SH replacement by other antimicrobial agents is mandatory in order to reduce the environmental impact and also to increase produce safety to the consumer (Gopal et al., 2010). These alternative methods can help to reduce the use of SH in disinfection, especially if used in combination. This can result in a synergistic effect, i.e., the combination of disinfectants can lead to a reduction of the necessary concentration, compared to when they are applied individually (Greco et al., 1995).

## 1.2. Objectives

The purpose of the present work was to bring further understanding on the critical aspects of contaminations along the MPV supply chain, namely: where the contamination occurs, and where and how the microorganisms colonise the MPV and processing equipment; how the microorganisms potentially resist during sanitising procedures and if alternative disinfection techniques would improve disinfection. Moreover, it is important to understand the biodiversity of a fresh vegetables processing plant. In order to develop efficient surface sanitation and food decontamination strategies, it is also crucial to identify the target microorganisms persisting along the processing chain. Characterize the effects of these microorganisms on the fresh vegetables properties and their susceptibility to antimicrobial agents is relevant to the quality of the produce and to the public health (Corbo et al., 2010).

As these work was integrated in an European Project (SUSCLEAN - Sustainable Cleaning and Disinfection in Fresh-Cut Food Industries), to achieve these goals the main aims were performed in the order presented:

**i.** Develop new decontamination and sanitation procedures to remove and control biofilms

In this task the potential of different chemicals was evaluated on biofilm removal and killing, in order to reduce the process equipment and the fresh produce contamination. Six chemical compounds (peracetic acid, chlorine dioxide, copper sulfate, hydrogen peroxide, sodium bicarbonate and vanillin) were tested individually and in combination with SH. The objective of these combinations was to try to reduce the total amount of

chlorinated residues and consequently the potential negative impact of decontamination on the health and the environment.

**ii.** Reduce the quantity of chlorine necessary to the disinfection process

Since chlorinated compounds are efficient, three chlorine-based disinfectants (neutral electrolyzed oxidizing water, chlorine dioxide and sodium dichloroisocyanurate) were tested and compared with SH. The aim was to find another disinfectant as efficient as SH but using a lower free chlorine concentration. Therefore, the main purpose was to reduce chlorine concentration, whilst ensuring food safety, sustainable practices and preserving fresh-cut food European quality and competitiveness.

**iii.** Isolate and identify microorganisms from a MPV plant

The microorganisms present in a typical MPV plant were isolated from the air, surfaces and produce. After the isolation procedures, the microorganisms were identified. With this task, the contamination scheme could be described: microorganism populations and colonisation patterns on equipment and produce surfaces. The main aim was to identify the persisting bacteria and to detect the different critical points along the process chain.

**iv.** Characterize the isolated microorganisms

The detailed characterization of the microbial isolates is critical to the evaluation of their potential effects on human health and in the final product. In this task, the purpose was to determine the critical microorganisms in the food processing stages. Biofilm-associated microorganisms play crucial roles in contamination, therefore it was important to distinguish which of the isolated microorganisms were more capable of forming biofilms (Kumar and Anand, 1998, Srey et al., 2013). These microorganisms should be eliminated, since if they are removed, the microflora in the surfaces of the equipment can be reduced. In this way, pathogenicity and/or virulence aspects of the isolated microorganisms were characterized taking in to account the following aspects: biofilm formation capability, aggregation potential, production of proteases, gelatinases and siderophores and quorum sensing inhibition (QSI). Besides, their resistance profile to selected in use antibiotics and disinfectants was also evaluated. The health concern is also a significant aspect to be studied, since the ultimate consumer is the possible impaired. As certain microorganisms can resist in the stomach acidic environment (Frank, 2001), it was important to perform tests to understand the microbial behavior under such conditions.

**v.** Comparison of a food isolate *vs* a reference strain

The search for alternative disinfectants and appropriate conditions that should be applied are usually done in a laboratory scale and with a reference or collection strain (Langsrud et al., 2003, Carpentier and Chassaing, 2004). Some bacteria survive the disinfection practices, which means that they have less susceptibility to the chemical agents (Langsrud et al., 2003). The most resilient microbial isolate obtained from the MPV industry studied, in terms of biofilm formation, was compared to a reference strain (*E. coli* CECT 434). The best disinfectant determined in task *i* was used and compared with SH action, using different methods. A bioreactor system, allowing multiple slides to obtain replicas, was used for biofilm formation, as well as 96-well microtiter plates. The susceptibility of both bacteria to selected disinfectants and antibiotics was compared.

**vi.** Develop of a new artificial lettuce medium

MPV can possibly potentiate the pathogenic growth, especially due to the high water content. It has been described by several authors (Berger et al., 2010, Taban and Halkman, 2011, Schikora et al., 2012, Posada-Izquierdo et al., 2016) that fresh produce can be considered a host or vehicle for the microorganisms. To understand how the produce affects microbial growth a new artificial medium, mimicking the lettuce chemical composition, was tested. The artificial medium developed was studied and compared to a commonly used medium (Mueller–Hinton broth (MHB)). The factors tested were microbial growth, biofilm formation and disinfectants susceptibility.

# 2

## LITERATURE REVIEW

This literature review provides updated information on the state of art of the available disinfection strategies alternative to chlorine that can be used in the fresh-cut industry. The use of combined methods to replace and/or reduce the use of chlorine are also reviewed.

## 2.1. Introduction

Fresh produce and MPV are consumed worldwide as they are important natural sources of essential nutrients. For the modern consumer, these products are necessary to maintain a healthy diet, and their fresh and nutritional status is largely recognized (Williams, 1995, Lampe, 1999, Randhawa et al., 2015). However, despite the increased awareness of food safety issues, the occurrence of foodborne disease outbreaks related to these products is constantly increasing (Gilbert and McBain, 2003, Ölmez and Kretzschmar, 2009). To increase the shelf-life and also enhance the microbial safety of these products, chlorine is commonly applied as hypochlorous acid and hypochlorite in the fresh-cut industry, as a disinfectant at concentrations varying between 50 and 200 ppm of free chlorine and for a maximum exposure time of 5 min (Rico et al., 2007, Goodburn and Wallace, 2013). It was verified that this is the maximum exposure time applied, since other works (Adams et al., 1989) found that "longer wash times (from 5 to 30 min) did not result in increased removal of microorganisms". The exposure time can also depend on the microorganism (Tirpanalan et al., 2011). Chlorine is widely used in the food industry (Sagong et al., 2011, Van Haute et al., 2013) due to its relatively low price, facility to apply and wide spectrum of antimicrobial effectiveness (Ramos et al., 2013). However, this disinfectant shows limited efficiency in reducing microbial loads (Yaron and Romling, 2014), as it can be easily inactivated by organic matter (Parish et al., 2003, Ramos et al., 2013), and its action is highly pH dependent (Ramos et al., 2013). Furthermore, this disinfectant can produce unhealthy by-products including carcinogenic and mutagenic chlorinated compounds, such as chloroform and other trihalomethanes, chloramines and haloacetic acids, when reacting with organic molecules (Legay et al., 2010, Bull et al., 2011). Also, it is corrosive and has been included in the indicative list of the Directive on Industrial Emissions (IPPC, 2007/0286 (COD)), aiming to reduce harmful industrial emissions across the EU, therefore benefiting the environment and human health (European Comission, 2007). Its use is already prohibited in some European countries (Belgium, Denmark, Germany and The Netherlands) (Ölmez and Kretzschmar, 2009, Bilek and Turantaş, 2013, Ramos et al., 2013, Fallik, 2014). Although disinfection with chlorine is widespread in the fresh-

cut industry, there is a global concern on developing alternative disinfection strategies to minimize its environmental and public health impacts (Gopal et al., 2010). Different methods to reduce and/or replace the use of chlorine have already been developed. Those include biological methods, alternative chemical compounds and physical technologies, or even the combination of methods (Gil et al., 2009, Ölmez and Kretzschmar, 2009, Otto et al., 2011, Bilek and Turantaş, 2013, Goodburn and Wallace, 2013, Fallik, 2014, Holah, 2014) (Figure 2.1). Most of these methods are recognized as environmentally friendly, and do not represent a potential risk to the health and safety of workers and consumers (Lado and Yousef, 2002, Fallik, 2014, Holah, 2014). These alternative methods (biological, chemical and physical) are applied according to each target: produce, food-contact surfaces and water (Table 2.1).

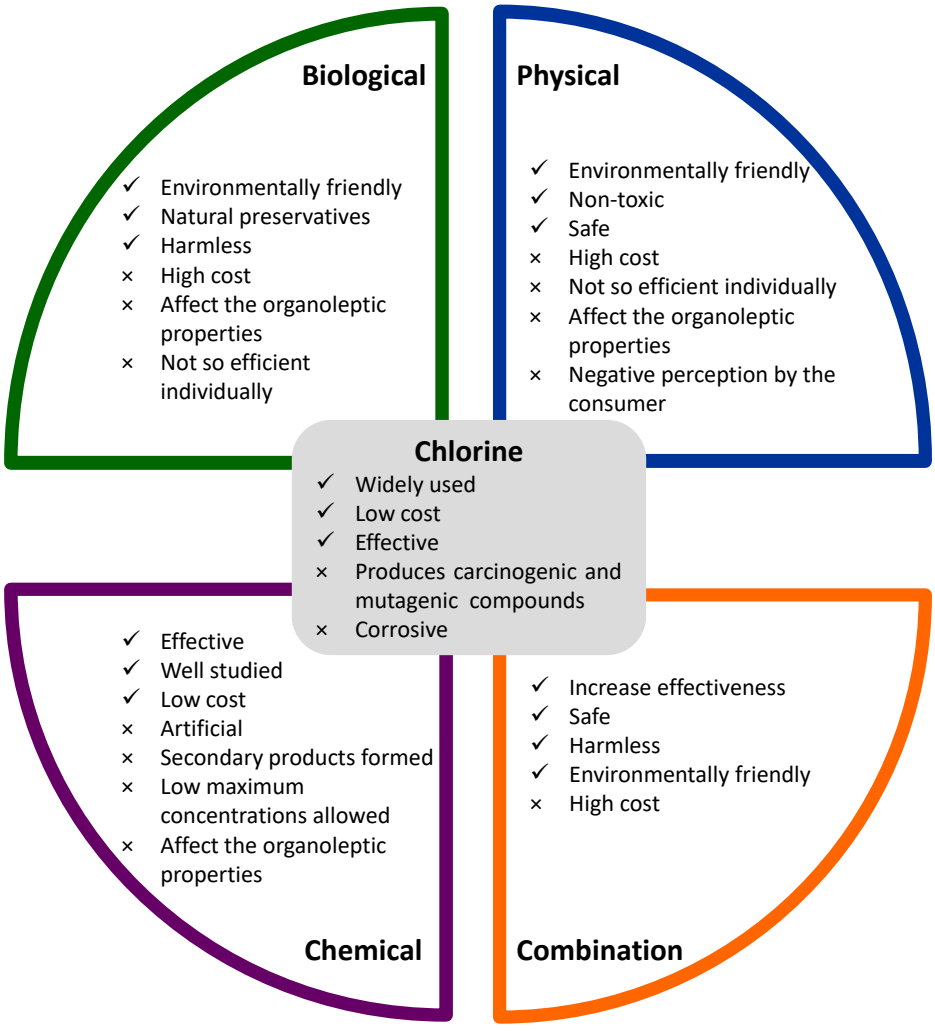


**Figure 2.1.** Schematic overview on the advantages and disadvantages of chlorine and the alternative methods of disinfection and/or decontamination (biological, physical, chemical and their combination).

**Table 2.1.** Alternative disinfection methods used in the fresh-cut food industry, applied on the food-contact surfaces, on the produce and on the water

| Target | Method | Results | Reference |
|---|---|---|---|
| Food contact surfaces | Nisin ($6.75\times10^{-3}$ ppm, 5 min, 20 ºC) | Reduction of 2.58 log CFU.cm$^{-2}$ of *L. monocytogenes* on SS surfaces | (Arevalos-Sánchez et al., 2012) |
| | Nisin ($6.75\times10^{-3}$ ppm, 20 min, 20 ºC) | Reduction of 1.92 log CFU.cm$^{-2}$ of *L. monocytogenes* on glass surfaces | (Arevalos-Sánchez et al., 2012) |
| | Lytic phage (phage ϕ S1) | Reduction of 80% of *Pseudomonas fluorescens* biofilm on SS surfaces | (Sillankorva et al., 2004) |
| | Carvacrol (500-1000 ppm, 1 hour) | Reduction of 7 log CFU of *Salmonella* sp. on PS and SS surfaces | (Soni et al., 2013) |
| | Carvacrol (300 ppm) | Reduction of 2-3 log CFU of bacteria (listeriae and salmonellae) on SS surfaces | (Knowles and Roller, 2001) |
| | CD (200 ppm) | Reduction of 4.42 log CFU of *B. cereus* on SS surfaces | (Kreske et al., 2006) |
| | CD ($5\times10^{4}$ ppm, 10 min) | Reduction of 4.14 log CFU.chip$^{-1}$ of *L. monocytogenes* biofilm | (Robbins et al., 2005) |
| | EOW (56 ppm of free chlorine, 5 min) | Reduction of 9 log CFU.cm$^{-2}$ of *L. monocytogenes* on SS surfaces | (Kim et al., 2001) |
| | NEOW (63 ppm of free chlorine, 1 min) | Reduction of 6 log CFU.cm$^{-2}$ of *E. coli*, *P. aeruginosa*, *Staphylococcus aureus* and *L. monocytogenes* on SS and glass surfaces | (Deza et al., 2005) |
| | NEOW (70 ppm of free chlorine, 3 min) | *L. monocytogenes* biofilms (on SS surfaces) completely inhibited | (Arevalos-Sánchez et al., 2013) |
| | Aqueous ozone (5.9 ppm, 1 min) | *B. subtilis* and *P. fluorescence* were completely eliminated from SS surfaces | (Khadre & Yousef, 2001) |
| | Sodium bicarbonate ($5\times10^{4}$ ppm, 1 min) | Reduction of 99.22% of feline calicivirus on food contact surfaces | (Malik and Goyal, 2006) |
| | US (40 kHz) | 30% of *E. coli* biofilm removal on SS | (Oulahal-Lagsir et al., 2003) |
| | α-amylase + Realco B (30 min) | Reduction of 2.98 log CFU.cm$^{-2}$ of *B. mycoides* on SS surfaces | (Lequette et al., 2010) |
| | Sodium bicarbonate ($2\times10^{4}$ ppm) + HP ($2\times10^{4}$ ppm), for 10 min | Reduction of 99.68% of feline calcivirus on food contact surfaces | (Malik and Goyal, 2006) |
| | US (40 kHz) + trypsin (7600 U.mL$^{-1}$) | 76% of *E. coli* biofilm removal on SS | (Oulahal-Lagsir et al., 2003) |

**Table 2.1.** (continued)

| Target | Method | Results | Reference |
|---|---|---|---|
| Fresh-cut produce | Nisin (50 ppm, 1 min) | Reduction of 2.20 and 4.35 log CFU of *L. monocytogenes* on mung bean and broccoli, respectively | (Bari et al., 2005) |
| | Lytic bacteriophages (UAB_Phi 20, UAB_Phi78, and UAB_Phi87) (60 min at room temperature) | Reduction of 3.9 and 2.2 log $CFU.g^{-1}$ for *S.* Typhimurium and *S.* Enteritidis, respectively, on lettuce | (Spricigo et al., 2013) |
| | Lytic *L. monocytogenes*-specific phages | Reduction of 2.0-4.6 log CFU of *L. monocytogenes* per melon sample | (Leverentz et al., 2003) |
| | Carvacrol (150 ppm) | Reduction of the total viable counts in 4.6 log $CFU.g^{-1}$ on kiwi | (Roller and Seedhar, 2002) |
| | Cinnamic acid (150 ppm) | Reduction of the total viable counts in 4.6 log $CFU.g^{-1}$ on kiwi | (Roller and Seedhar, 2002) |
| | Oregano oil (25, 40 and 75 ppm, at 5, 10, 15 and 20 min) | Reduction of 1.92 log $CFU.g^{-1}$ of *S.* Typhimurium on lettuce | (Gündüz et al., 2010) |
| | CD (10 ppm, 5 min) | Reduction of 1.2 log $CFU.g^{-1}$ of *E. coli* O157:H7 on lettuce | (Singh et al., 2002a) |
| | CD (100 ppm) | Reduction of 3.5-4.0 log $CFU.g^{-1}$ of total bacterial and coliform counts on lettuce | (Chung et al., 2011) |
| | CD (100 ppm) | Reduction of 1.25 log $CFU.g^{-1}$ of *E. coli* O157:H7 on lettuce | (Keskinen et al., 2009) |
| | AcEOW (pH 2.6, at 50 ppm (free chlorine), 2 min) | Reduction of 1 log $CFU.g^{-1}$ of *E. coli* O157:H7 on lettuce | (Keskinen et al., 2009) |
| | AcEOW (pH 2.06, at 37.5 ppm (free chlorine), 1 min) | Reduction of 4.45 log $CFU.g^{-1}$ of *E. coli* O157:H7 on green onions | (Park et al., 2008) |
| | NEOW (89 ppm (free chlorine), 5 min treatment) | Reduction of 6 log $CFU.mL^{-1}$ of *E. coli* O157:H7, *S.* Enteritidis and *L. monocytogenes* on tomatoes | (Deza et al., 2003) |
| | NEOW (20 ppm (free chlorine), 10 min) | Reduction of 6 log $CFU.mL^{-1}$ of *E. coli*, *S.* Typhimurium, *S. aureus*, *L. monocytogenes* and *Enterococcus faecalis*, on lettuce | (Guentzel et al., 2008) |

**Table 2.1.** (continued)

| Target | Method | Results | Reference |
|---|---|---|---|
| Fresh-cut produce | NEW (50 ppm free chlorine) | Reduction of 1-2 log CFU.mL$^{-1}$ of *E. coli* O157:H7, *Salmonella, L. innocua* and *Erwinia carotovora* on lettuce | (Abadias et al., 2008) |
| | HP ($3\times10^4$ ppm, 5 min) | Reduction of 1.6 log CFU.g$^{-1}$ reduction of *E. coli* O157:H7 on baby spinach leaves | (Huang et al., 2012) |
| | HP ($5\times10^4$ ppm, 2 min) | Reduction of 2.0-3.5 log CFU.cm$^{-2}$ of *L. monocytogenes* from melon surfaces | (Ukuku and Fett, 2002) |
| | HP ($2\times10^4$ ppm) | Reduction of 1.5 log CFU.g$^{-1}$ of *E. coli* O157:H7 on baby spinach leaves | (Huang and Chen, 2011) |
| | Citric acid ($5\times10^3$ to $1\times10^4$ ppm, at 20 ºC for 1 to 5 min) | Reduction of 1 log CFU.cm$^{-2}$ of *Listeria monocytogenes* from lettuce | (Samara and Koutsoumanis, 2009) |
| | Acetic acid (20 ºC for 2-5 min) | Reduction 2.2 and 1.3 log CFU.g$^{-1}$ of *E. coli* and *L. monocytogenes* respectively, from lettuce | (Akbas and Olmez, 2007) |
| | Lactic acid (20 ºC for 2-5 min) | Reduction of 2.8 and 2.1 log CFU.g$^{-1}$ of *E. coli* and *L. monocytogenes*, respectively, from lettuce | (Akbas and Olmez, 2007) |
| | PA (120 ppm) | Reduction of the microbial load in 1.2 log CFU.g$^{-1}$ on fresh-cut iceberg lettuce | (Vandekinderen et al., 2009) |
| | PA (40 ppm, 5 min) | Reduction of 0.99 log CFU.g$^{-1}$ of *S.* Typhimurium on lettuce | (Ge et al., 2013) |
| | Propionic acid ($1\times10^4$ ppm, 10 min) | Reduction of 0.93-1.52 log CFU.g$^{-1}$ of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* on organic fresh lettuce | (Park et al., 2011) |
| | Acetic acid ($1\times10^4$ ppm, 10 min) | Reduction of 1.13-1.74 log CFU.g$^{-1}$ of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* on organic fresh lettuce | (Park et al., 2011) |
| | Lactic acid ($1\times10^4$ ppm, 10 min) | Reduction of 1.87-2.54 log CFU.g$^{-1}$ of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* on organic fresh lettuce | (Park et al., 2011) |
| | Malic acid ($1\times10^4$ ppm, 10 min) | Reduction of 2.32-2.98 log CFU.g$^{-1}$ of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* on organic fresh lettuce | (Park et al., 2011) |

**Table 2.1.** (continued)

| Target | Method | Results | Reference |
|---|---|---|---|
| Fresh-cut produce | Citric acid ($1\times10^4$ ppm, 10 min) | Reduction of 1.85-2.86 log CFU.g$^{-1}$ of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* | (Park et al., 2011) |
| | Benzalkonium chloride (2 ppm) | Growth of *B. cereus*, *S. aureus* and *E. coli* (isolated from fresh vegetables) completely inhibited | (Park et al., 2013) |
| | Cetylpyridinium chloride ($5\times10^3$ ppm) | Reduction of 3.70, 3.15 and 1.56 log CFU.g$^{-1}$ for *L. monocytogenes*, *S.* Typhimurium and *E. coli* O157:H7, respectively, from broccoli, cauliflower, and radishes | (Wang et al., 2001) |
| | Aqueous ozone (5 ppm, 5 min) | Reduction of 1.8 log CFU of *Shigella sonnei* from shredded lettuce | (Selma et al., 2007) |
| | Gaseous ozone (5-10 ppm) | Reduction 1.8 log CFU.g$^{-1}$ of *E. coli* O157:H7 on spinach leaves | (Vurma et al., 2009) |
| | Sodium bicarbonate ($2\times10^4$ to $4\times10^4$ ppm, 150 seconds at room temperature) | Reduction of 50% of blue mold by *Penicillium italicum* in citrus | (Palou et al., 2001) |
| | UV lamp (254 nm) | Reductions of 1.75, 1.27, 1.39 and 1.21 log CFU.g$^{-1}$ of *E. coli*, *L. innocua*, *S.* Enteritidis and *S. aureus*, respectively, on lettuce | (Birmpa et al., 2013) |
| | UV lamp (253.7 nm, 60 min) | Reduction of 1.7 log CFU.g$^{-1}$ of *E. coli* on lettuce | (Bermúdez-Aguirre and Barbosa-Cánovas, 2013) |
| | UV-C lamp (254 nm, irradiation fluency of 450 mJ.cm$^{-2}$, 5 min) | Reduction of 2.28 log CFU.g$^{-1}$ of *S.* Typhimurium on lettuce | (Ge et al., 2013) |
| | US (32-40 kHz, 10 min) | Reduction of 1.5 log CFU.g$^{-1}$ of *S.* Typhimurium on cut iceberg lettuce | (Seymour et al., 2002) |
| | US (37 kHz, 30 min) | Reduction of 2.30, 5.72 and 1.88 log CFU.g$^{-1}$ of *E. coli*, *S.* Enteritidis, *L. innocua*, respectively, on lettuce. | (Birmpa et al., 2013) |
| | US (40 kHz, at 23 ºC, for 30 min) | Reduction of 1.08 log CFU.g$^{-1}$ of *E. coli* O157:H7 on broccoli seeds | (Kim et al., 2006) |

**Table 2.1.** (continued)

| Target | Method | Results | Reference |
|---|---|---|---|
| Fresh-cut produce | Irradiation (0.55 kGy) + chlorine (200 ppm) | Reduction of 5.4 log CFU.g$^{-1}$ of *E. coli* O157:H7 on shredded iceberg lettuce | (Foley et al., 2002) |
| | Heating (50 ºC) + HP (2×10$^4$ ppm) | Reduction of 2.2 log CFU.g$^{-1}$ of *E. coli* O157:H7 on baby spinach | (Huang and Chen, 2011) |
| | Heating (50 ºC, 1 min) + chlorine (100 ppm) | Reduction of 2.0 log CFU.g$^{-1}$ of total microbial populations on fresh-cut iceberg lettuce | (Delaquis et al., 2004) |
| | US (32-40 kHz) + chlorine (25 ppm) (10 min treatment) | Reduction of 2.7 log CFU.g$^{-1}$ of *S.* Typhimurium from cut lettuce | (Seymour et al., 2002) |
| | US (40 kHz) + lactic acid (2×10$^4$ ppm), for 5 min at 20 ºC | Reduction of 2.75, 2.71 and 2.50 log CFU.g$^{-1}$ of *E. coli* O157:H7, *S.* Typhimurium and *L. monocytogenes*, respectively, on organic lettuces | (Sagong et al., 2011) |
| | CD (20-40 ppm) + US (170 kHz) | Reduction of 2.6 and 1.8 log CFU.g$^{-1}$ of *Salmonella* spp. and *E. coli* O157:H7 on lettuce | (Huang et al., 2006) |
| | CD (40 ppm) + ozone (5 ppm) (5 min treatment) | Reduction of 2.17 log CFU.g$^{-1}$ of total bacterial count on turnip greens | (Ibrahim et al., 2008) |
| | Copper (40 ppm) + lactic acid (2×10$^3$ ppm) | Reduction of 3.93 log CFU.cm$^{-2}$ of *E. coli* O157:H7 on lettuce surface | (Gyawali et al., 2011) |
| | AlEOW (100 ppm) + citric acid (1×10$^4$ ppm) at 50 ºC | Reduction of 3.99 log CFU.g$^{-1}$ and 4.19 log CFU.g$^{-1}$ of *L. monocytogenes* and *E. coli* O157:H7, respectively, on cabbage | (Rahman et al., 2010) |
| | Citric acid (1×10$^4$ ppm) + ozonated water (3 ppm), for 1 min | Reduction of 2.31 and 1.84 log CFU.g$^{-1}$ of *E. coli* O157:H7 and *L. monocytogenes*, respectively, on lettuce | (Yuk et al., 2006) |
| | UV-C (254 nm, irradiation fluency of 900 mJ.cm$^{-2}$, 10 min) + chlorine (200 ppm, 10 | Reduction of 2.40 log CFU.g$^{-1}$ of *S.* Typhimurium on lettuce | (Ge et al., 2013) |
| | UV-C (254 nm, irradiation fluency of 900 mJ.cm$^{-2}$, 10 min) + PA (80 ppm, 10 min) | Reduction of 2.52 log CFU.g$^{-1}$ of *S.* Typhimurium on lettuce | (Ge et al., 2013) |

**Table 2.1.** (continued)

| Target | Method | Results | Reference |
|---|---|---|---|
| Washing water | Ozone (2 ppm, 10 min) | Reduction of 1.56 log CFU.mL$^{-1}$ of *B. subtilis* | (Rosenblum et al., 2012) |
| | US (20 kHz, 53 min) | Reduction of 4.4 log CFU.mL$^{-1}$ of *E. coli* O157:H7 on fresh-cut vegetables wash water | (Elizaquível et al., 2012) |
| | UV and ozone (60 min) | Microbial reduction of 6.6 log CFU.mL$^{-1}$ in escarole wash water | (Selma et al., 2008) |
| | QACs (n-alkyl dimethyl benzyl ammonium chloride sulfosuccinate dioetil and urea) (100 and 200 ppm, 30 and 120 seconds) | Reduction of 99.99% of *E. coli* and *S. aureus* (for 100 and 200 ppm, for 30 and 120 seconds, for low and high turbidity), except for *E. coli* with high turbidity in the disinfection process of 100 ppm at 30 and 120 seconds (20.78% and 87.94%, respectively). | (Chaidez et al., 2007) |

## 2.2. Biological-based methods

### 2.2.1. Bacteriocins

One possibility to prevent the growth of both spoilage and pathogenic microorganisms is the exploitation of their competition with other microorganisms, typically with beneficial ones (Ramos et al., 2013). Lactic acid bacteria (LAB) are an example of such beneficial microorganisms, having GRAS (Generally Recognized as Safe) status. Furthermore, LAB produce antimicrobial compounds, such as organic acids and bacteriocins, which can be used as antimicrobials (Rodgers, 2001, 2008). A well-known example of such compound is the bacteriocin nisin. Nisin acts on the cell membrane forming pores that result in cell death (Bari et al., 2005, Arevalos-Sánchez et al., 2012). This natural preservative is produced by *Lactococcus lactis* and is effective mainly against Gram-positive bacteria (Hansen, 1994, Davies and Delves-Broughton, 1999, Arevalos-Sánchez et al., 2012, Magalhães et al., 2014). The Gram-negative bacteria are not affected by this peptide due to their outer membrane. Nevertheless, this drawback can be overcome by exposing the cells to chelating agents or osmotic shock that destabilize the outer membrane before the use of nisin (Delves-Broughton, 2005, Angiolillo et al., 2014). The major advantage on the use of nisin as a disinfectant is the fact that it is harmless for the consumer and is already used as a food preservative (O'Keeffe and Hill, 1999). With regard to fresh produce, Bari et al. (2005) used nisin at 50 ppm for 1 min on mung bean and broccoli and achieved the reduction of *L. monocytogenes* by 2.20 and 4.35 log CFU (colony forming units).$g^{-1}$, respectively. The disinfecting efficacy observed with 1 min contact time was higher for broccoli. Allende et al. (2007) incorporated nisin and coagulin individually and combined in tryptic soy agar (TSA) plates and obtained reductions of *L. monocytogenes* of 1.0-1.5 log CFU after 48 hours of storage at 4 ºC. Bacteriocins have also been used to disinfect stainless steel (SS) and glass surfaces. Arevalos-Sánchez et al. (2012) used nisin at $6.75\times10^{-3}$ ppm for 5 min at 20 ºC to eliminate *L. monocytogenes* from SS and achieved a 2.58 log CFU.$cm^{-2}$ reduction. Applying the same concentration for 20 min in glass surfaces resulted in 1.92 log CFU.$cm^{-2}$ reduction, which means that glass surfaces contaminated with *L. monocytogenes* are probably more difficult to clean. The search for new chemicals derived from the microbial metabolism will certainly provide new disinfectants to be applied in the food industry. In fact, it is estimated that less than 1% of the bacteria are culturable with current methods, which lead to an underestimation

of the microbial diversity and a lack of knowledge on the microbial metabolites available in nature (Lasa et al., 2014, Ling et al., 2015).

### 2.2.2. Bacteriophages

The use of bacteriophages as preservatives and disinfecting agents is not a recent application, and the interest on these agents has increased throughout the years (Hughes et al., 1998, Kudva et al., 1999, Sharma et al., 2005, Spricigo et al., 2013). Bacteriophages are viruses that infect bacteria causing their lysis (Simões et al., 2010b). The main advantages on the use of lytic bacteriophages to destroy unwanted bacteria are their: i) specificity; ii) effective mode of action (Spricigo et al., 2013); iii) availability (Hughes et al., 1998); and iv) reduced effects on the organoleptic properties of the products (Sharma et al., 2005). Spricigo et al. (2013) used three different lytic bacteriophages (UAB_Phi 20, UAB_Phi78, and UAB_Phi87) to control *S.* Typhimurium and *S.* Enteritidis on lettuce. The treatment was performed for 60 min at room temperature and the reduction achieved was 3.9 and 2.2 log CFU.g$^{-1}$ for *S.* Typhimurium and *S.* Enteritidis, respectively (Spricigo et al., 2013). Although significant CFU reduction was observed, the treatment time was too long making this strategy impractical for the fresh-cut industry. Kudva et al. (1999) demonstrated that the combination of these three phages was capable to cause the lysis of *E. coli* O157:H7 at both 4 and 37 ºC. The use of phages was also studied to inactivate *L. monocytogenes* on melons by Leverentz et al. (2003). These authors obtained a reduction of 2.0–4.6 log CFU per sample (Leverentz et al., 2003). Sillankorva et al. (2004) used a lytic phage (phage ϕ S1) on SS coupons and were able to remove 80% of *P. fluorescens* biofilms. These evidences on the efficacy of bacteriophages to control spoilage and pathogenic microorganisms are promising. Nevertheless, further research is required to increase the antimicrobial action of bacteriophages and to reduce the contact time.

### 2.2.3. Enzymes

Enzymes can attack directly the biofilms interfering with their development process, catalyze the formation of antimicrobials, interfere with quorum sensing (QS) events, or even destroy a mature biofilm (Simões et al., 2010b, Thallinger et al., 2013). Enzymes mainly target the extracellular polymeric matrix which surrounds the biofilm cells and influences the shape of biofilm structure and its resistance to shear forces (Lequette et al., 2010). Therefore, enzymes can be considered as an alternative method to conventional

chemical disinfectants to remove biofilms from produce leaves and/or from abiotic surfaces. Like the bacteriophages the application of enzymes requires prolonged contact times to be effective in biofilm control. Another disadvantage on the use of enzymes for biofilm removal is the fact that the EPS are heterogeneous. Therefore, the use of pure enzymes do not guarantee complete biofilm elimination. In fact, they should be used as a mixture or combined with other treatments, particularly antimicrobial agents (Augustin et al., 2004, Lequette et al., 2010). These formulations are mostly applied for the disinfection of food-contact surfaces (Thallinger et al., 2013). Another drawback on the use of enzymes is their relative high cost (Augustin et al., 2004, Simões et al., 2010b, Thallinger et al., 2013).

Typical applications of enzymes on biofilm removal are the use of proteases in pipelines and the removal of proteins from contact lenses (Augustin et al., 2004). Some studies have been developed to remove bacterial biofilms found in the food industry particularly on SS surfaces, with the use of proteolytic enzymes (Lequette et al., 2010). Lequette et al. (2010) used a buffer with an anionic surfactant mixed with α-amylase during 30 min and found that this treatment reduced the biofilm of *Bacillus mycoides* on SS surfaces by 2.98 log $CFU.cm^{-2}$. Augustin et al. (2004) obtained *P. aeruginosa* reductions of 4 log $CFU.mL^{-1}$ after treatment with enzymatic solutions (Pandion, Resinase, Spezyme and Paradigm used individually) for 30 min.

### 2.2.4. Phytochemicals

Plants have the ability to produce secondary metabolites (phytochemicals) with antimicrobial properties against several microorganisms, including pathogens (Cowan, 1999, Belletti et al., 2008). These metabolites are divided into diverse chemical classes, such as alkaloids, essential oils, phenolics, polyphenolics, polyacetylenes, lectins and peptides (Cowan, 1999, Borges et al., 2013); and subclasses: isothiocyanates, terpenoids, thiosulfinates, phenolic acids, simple phenols, terpenoids, polyamines, polyketides, quinones, flavones, flavonoids, flavonols, etc (Cowan, 1999, Newman et al., 2000, Simões et al., 2012). Many of these molecules have GRAS status and are widely used in the food industry (Singh et al., 2002b). Given their great variability, the mode of action of phytochemicals is quite diverse. The most common effect involves the increase of the cell membrane permeability leading to the leakage of intracellular compounds (Singh et al., 2002b, Tiwari et al., 2009). Such promising phytochemicals are the essential oils, which are mostly used as flavoring agents for foodstuffs and in perfumery; however, they

are also used as antimicrobial agents in the food industry (Cowan, 1999, Borges et al., 2013). Carvacrol is the main component of the essential oil of oregano and thyme. This phytochemical was used by Roller and Seedhar (2002) to disinfect kiwi at a concentration of 150 ppm and resulted in 4.6 log $CFU.g^{-1}$ reduction of the total viable counts. It has also been used on food-contact surfaces. Soni et al. (2013) used concentrations from 500 to 1000 ppm of carvacrol (with an exposure time of 1 hour) and reduced 7 log CFU of *Salmonella* sp. on polystyrene (PS) and SS surfaces. Other authors (Knowles and Roller, 2001) used carvacrol (300 ppm) and were able to eliminate 2-3 log CFU of adhered bacteria (listeriae and salmonellae) from SS. Gündüz et al. (2010) used oregano oil to inactivate *S.* Typhimurium on lettuce at three different concentrations (25, 40 and 75 ppm) and for four different contact times (5, 10, 15 and 20 min). The treatments did not exceed a reduction of 1.92 log $CFU.g^{-1}$ regardless the condition tested (Gündüz et al., 2010). Those authors also found that the efficacy of 75 ppm of oregano oil was comparable to 50 ppm of chlorine in the disinfection of lettuce (Gündüz et al., 2010). Phenolic compounds are the most important and abundant class of phytochemicals (Borges et al., 2013). Cinnamic acid was also used in the study of Roller and Seedhar (2002) with antimicrobial efficacy similar to carvacrol. Even if they are green chemicals, phytochemicals can alter the organoleptic properties of the fresh produce (Roller and Seedhar, 2002, Belletti et al., 2008, Kentish and Ashokkumar, 2011) and the high cost of some of those products can limit their current use at industrial scale (Roller and Seedhar, 2002). In order to have a practical application in the food industry with higher CFU reductions and lower contact times, the use of phytochemicals has to be further studied on the search for new and more effective products and/or on their potential synergistic effects when combined with other methods.

## 2.3. Chemical-based methods

### 2.3.1. Calcium lactate

Calcium is usually used to maintain the firmness of fresh produce during storage (Rico et al., 2006) since this is able to interact with pectin, maintaining the structure of the cell wall, while lactate has antimicrobial properties (Martín-Diana et al., 2005a, Martín-Diana et al., 2006). Calcium lactate has also the advantage of not giving an off-flavor and bitterness to the products (Martín-Diana et al., 2005b). However, the number of research studies with this product is scarce. One such study by Martín-Diana et al. (2005b)

concluded that using a solution of $3\times10^4$ ppm of calcium lactate resulted in the same reduction of mesophilic counts in fresh-cut lettuce as a solution of $12\times10^4$ ppm of SH, while this treatment was considered acceptable by the consumer.

### 2.3.2. Chlorine dioxide

The use of chlorine dioxide (CD) in the fresh-cut industry was studied by Tomás-Callejas et al. (2012) and compared with SH. The authors found that CD: i) has a higher oxidation capacity; ii) does not react with nitrogen or ammonia to form harmful by-products (Rico et al., 2007); iii) has lower reactivity with organic matter; iv) is less corrosive than SH (Ölmez and Kretzschmar, 2009); and v) can inhibit enzymatic browning (Chen et al., 2010). However, the use of CD also presents some disadvantages: i) its maximum allowed concentration is quite low (3 ppm) (21CFR173.300, 2014); ii) it is unstable since it is explosive and has to be generated on site (Gómez-López et al., 2009); iii) its antimicrobial efficiency is pH dependent (Ölmez and Kretzschmar, 2009); and iv) it is readily degraded when exposed to sunlight (Tomás-Callejas et al., 2012). CD can be produced by two different ways: the reaction of an acid with sodium chlorite, or the reaction of sodium chlorite with chlorine gas (Ölmez and Kretzschmar, 2009) and as thus this can be obtained in either aqueous or gaseous forms, respectively (Macnish et al., 2008). Although it is a disinfectant accepted by FDA (Food and Drug Administration) (21CFR173.300, 2014), its use is still under assessment by the EU in the Regulation No 1062/2014 (EFSA, 2015). The mode of action of CD is related to its penetration through the cell membrane and the subsequent inhibition of metabolic functions (Joshi et al., 2013). López-Gálvez et al. (2010) found that CD is as effective as SH with the advantage of not forming trihalomethanes. Singh et al. (2002a) used CD at 10 ppm for 5 min and obtained 1.2 log $CFU.g^{-1}$ reduction of *E. coli* O157:H7 on lettuce. In the work of Mahmoud and Linton (2008) treatment with CD gas significantly reduced selected pathogens and inherent microorganisms on lettuce; however, a negative impact on the visual leaf quality was observed. Chung et al. (2011) evaluated the bactericidal efficacy of CD and SH solution for six types of fresh-cut vegetables and fruits and found that 100 ppm of CD solution reduced 3.5-4.0 log CFU per g of lettuce, carrot and tomato which was better than the action of the SH solution. Using the same concentration of CD, Keskinen et al. (2009) achieved 1.25 log $CFU.g^{-1}$ reduction of *E. coli* O157:H7 on lettuce. Trinetta et al. (2011) studied gaseous CD and found no chemical residues in the fresh products tested (tomatoes, lettuce, cantaloupe, alfalfa sprouts, oranges, apples and strawberries). Kreske

et al. (2006) reduced the biofilms of *B. cereus* in 4.42 log CFU per SS coupon applying 200 ppm of CD. This sanitizer has also been used to remove *L. monocytogenes* biofilms by Robbins et al. (2005). These authors used $5\times10^4$ ppm CD for 10 min and achieved a reduction of 4.14 log $CFU.chip^{-1}$. Apparently, CD is a disinfectant equally effective compared to SH but at lower concentrations and similar contact times.

### 2.3.3. Copper compounds

Microorganisms need copper at very low concentrations as a micronutrient, mainly used as a cofactor for certain enzymes and metalloproteins. However, at high concentrations it alters the membrane integrity, inactivates enzymes and produces free radicals causing cell death (Ibrahim et al., 2008). Copper compounds have been mainly used as fungicides, acting as a mediator of hydroperoxide, inducing cell damage (Costa, 2008). This process is irreversible and affects the respiratory chain, with the consequent loss of viability (Cerioni et al., 2009). The major limitation on the use of copper is related with its toxicity. Copper concentrations ranging from 0.6 to 2.4 ppm have been reported as 96 h $LC_{50}$ median lethal concentration values for juvenile *Penaeus monodon* (Chen and Lin, 2001). Copper is usually used in combination with other products such as lactic acid (Ibrahim et al., 2008, Gyawali et al., 2011), SH combined with ultrasounds (Rodgers and Ryser, 2004), SH and hydrogen peroxide (Cerioni et al., 2009, Cerioni et al., 2010, Cerioni et al., 2013). These combinations demonstrated to increase significantly the antimicrobial effects compared to the use of these products alone.

### 2.3.4. Electrolyzed oxidizing water

Electrolyzed oxidizing water (EOW) is a relatively new technology applied in the food industry. EOW, also known as activated water, is formed by the electrodyalisis of a sodium chloride solution in an electrolysis chamber with an anode and a cathode separated by a membrane (Deza et al., 2003, Demirci and Bialka, 2010, Cheng et al., 2012). To produce EOW, a salt (NaCl) diluted solution and current are passed through the chamber dissociating the solution into two separated streams: acid EOW (AcEOW) and alkaline EOW (AlEOW) (Ongeng et al., 2006, Hricova et al., 2008). The acid solution (pH between 2.5 and 3.5) is formed at the anode and it comprises HCl, HOCl, $Cl_2$, $OCl^-$, and $O_2$ and it also has a high oxidation-reduction potential (ORP) - between 1000 and 1200 mV. This solution is antimicrobial and with a mode of action similar to chlorine (DNA mutations, disruption of cell proteins and enzymes). Additionally and due to its

acidity, the cell membrane can be disrupted and the action of hypochlorous acid is facilitated (Huang et al., 2008, Demirci and Bialka, 2010). The alkaline solution (pH between 10 and 11.5) is produced at the cathode and is composed by hydroxyl ions, which can react with sodium ions forming sodium hydroxide (Hricova et al., 2008, Cheng et al., 2012). This alkaline solution works as a detergent and has a negative ORP (-800 to -900 mV) (Cheng et al., 2012). The neutral EOW (NEOW) (pH of 7 and an ORP of 700 mV) can be formed by the mixture of these two solutions (Cheng et al., 2012). In fact, the existence of a separating membrane is not mandatory and the anode and cathode solutions can be mixed inside the electrolysis cell. This method can be advantageous as the absence of the membrane avoids the occurrence of fouling, while the combined solution has advantages (Demirci and Bialka, 2010). NEOW is not so aggressive to the food-contact surfaces and is more stable than AcEOW, as chlorine decay occurs at low pH (Deza et al., 2003, Abadias et al., 2008, Cheng et al., 2012). NEOW can be used to disinfect food-contact surfaces and decontaminate the produce as it does not change the color or the appearance of the produce due to the neutral pH of the solution (Ayebah and Hung, 2005, Rico et al., 2008b). This method is environmentally friendly since it only uses NaCl and water to produce the chemical solution; there are no problems on handling the solution; and when this solution comes in contact with organic matter, or when diluted with tap water, it becomes water and can be safely discarded (Huang et al., 2008, Aday, 2016). Moreover, its use has already been approved by the FDA at a maximum concentration of 200 ppm (Food and Drug Administration, 2013). According to Sakurai et al. (2003) it has been used to disinfect digestive endoscopes between patients, being safe for the human body and for the environment. However, it is recommended to be produced in a ventilated place as the generation process leads to the production of $Cl_2$ and $H_2$. Furthermore, the equipment used for electrolysis is expensive as it is still not widely distributed and used (Cheng et al., 2012).

In terms of surface disinfection, several works have been performed mainly on SS surfaces. Arevalos-Sánchez et al. (2013) observed that *L. monocytogenes* biofilms on SS were completely inhibited after 3 min of contact time with NEOW at 70 ppm of free chlorine. Kim et al. (2001) reduced *L. monocytogenes* biofilms on SS surfaces by 9 log $CFU.cm^{-2}$ after 5 min of treatment with EOW at 56 ppm of free chlorine. Deza et al. (2005) studied the disinfection of both SS and glass surfaces with NEOW at 63 ppm of free chlorine for the reduction of *E. coli*, *Pseudomonas aeruginosa*, *Staphylococcus*

*aureus* and *L. monocytogenes*, reaching 6 log CFU.cm$^{-2}$ after 1 min of treatment. EOW was also studied for the decontamination of lettuce. Park et al. (2008) observed that using AcEOW (pH of 2.06 and 37.5 ppm of free chlorine) for 1 min reduced *E. coli* O157:H7 by 4.45 log CFU.g$^{-1}$ on green onions. Keskinen et al. (2009) used AcEOW (pH 2.6) at 50 ppm of free chlorine to reduce *E. coli* O157:H7 on lettuce. After 2 min treatment 1 log CFU.g$^{-1}$ reduction was achieved (Keskinen et al., 2009). Deza et al. (2003) in their study with tomatoes, found that NEOW was adequate to reduce *E. coli* O157:H7, *S.* Enteritidis and *L. monocytogenes* by 6 log CFU.mL$^{-1}$, following 5 min exposure to the disinfectant at 89 ppm of free chlorine, without affecting the organoleptic properties of the fresh product. Guentzel et al. (2008) obtained similar results (6 log CFU.mL$^{-1}$ reduction) using lettuce contaminated with *E. coli*, *S.* Typhimurium, *S. aureus*, *L. monocytogenes* and *Enterococcus faecalis*, following 10 min exposure time to NEOW at 20 ppm of free chlorine. Abadias et al. (2008) concluded that the use of NEOW (at 50 ppm of free chlorine) was equally effective as decontaminating lettuce with 120 ppm of chlorine solution, obtaining 1-2 log CFU.mL$^{-1}$ reduction of *E. coli* O157:H7, *Salmonella*, *L. innocua* and *Erwinia carotovora*. Aday (2016) also found that the browning effect caused by 25 ppm of EOW was very low. The application of NEOW is recommended to reduce the chlorine concentration (Abadias et al., 2008), since the efficiency is higher and the free chlorine content is lower. Further studies are required to characterize the chemical species formed during the generation of NEOW, their antimicrobial activity, stability and interaction with organic matter. There are evidences showing that in the presence of organic matter NEOW generates lower amounts of organochlorinated molecules than SH (Ayebah et al., 2006).

### 2.3.5. Hydrogen peroxide

Hydrogen peroxide (HP) is an oxidizer that can form cytotoxic species. The formation of these cytotoxic species is what assures its antimicrobial properties (Rico et al., 2007, Ölmez and Kretzschmar, 2009, Rahman et al., 2010) which can be either bactericidal or bacteriostatic (Brul and Coote, 1999, Ölmez and Kretzschmar, 2009), depending on the concentration, pH and temperature (Beuchat, 1998). This disinfectant can be applied on food-contact surfaces (Rico et al., 2007). However, according to Van Haute et al. (2015b) the use of HP cannot avoid the cross-contamination which can still occur in the vegetables washing water, as its decomposition is fast and the disinfection kinetics is slow. Another disadvantage is the browning effects that HP can cause to the vegetables, particularly to

lettuce (Beuchat, 1998, Rico et al., 2007, Ölmez and Kretzschmar, 2009). To overtake this aspect this chemical must be added in combination with a suitable anti-browning compound (Ölmez and Kretzschmar, 2009), such as sodium erythorbate (Sapers et al., 2001). Although HP has a GRAS status, its use in fresh produce decontamination is not allowed by FDA (Ölmez and Kretzschmar, 2009). Huang et al. (2012) used $3\times10^4$ ppm of HP to decontaminate baby spinach leaves for 5 minutes and obtained 1.6 log $CFU.g^{-1}$ reduction of *E. coli* O157:H7. Huang and Chen (2011) achieved similar log CFU reduction (1.5 log $CFU.g^{-1}$) of *E. coli* O157:H7 on the same product, but with a lower concentration of HP ($2\times10^4$ ppm). Using a higher concentration of HP ($5\times10^4$ ppm) for 2 minutes, Ukuku and Fett (2002) achieved a reduction of 2.0-3.5 log $CFU.cm^{-2}$ of *L. monocytogenes* on melon surfaces. Despite the fact that the concentrations used are very high, it is an environmental friendly disinfectant, as it is quickly decomposes into water and oxygen in the presence of catalase (an enzyme commonly found in plants); furthermore it is colorless and non-corrosive (St. Laurent et al., 2007, Fallik, 2014).

### 2.3.6. Ozone

Ozone ($O_3$) is produced as a gas that can be dissolved in water. When it is used in the dissolved form, only a small concentration (1-5 ppm) is needed to exert antimicrobial activity. However, higher concentrations are required when it is used as a gas, since the humidity of the air affects its penetration into the cells and the consequent disinfection process (Chauret, 2014, Horvitz and Cantalejo, 2014). It is a strong oxidizer with a high bactericidal potential (Foong-Cunningham et al., 2012). Furthermore, it spontaneously decomposes to a non-toxic product, $O_2$ (Kim et al., 2003, Atungulu and Pan, 2012). Nevertheless, its use has some disadvantages: i) it is unstable and rapidly decomposes (Chawla et al., 2012); ii) it can become very toxic (Chauret, 2014), as it can affect the respiratory tract and cause irritation to the eyes and throat (Artés et al., 2009); iii) its use is sensitive to the presence of organic matter; iv) it has to be generated on site (Chauret, 2014); v) it is not suitable to be used on the produce, as it can affect its physicochemical properties (Foong-Cunningham et al., 2012); and vi) it is potentially corrosive to the equipment (Sapers, 2009). However, ozone was already approved by FDA to be used on the food industry (21CFR173.368, 2014). In fact, it has been used as a decontaminant for the produce and disinfectant for the process water (Foong-Cunningham et al., 2012) and food-contact surfaces (Chauret, 2014). Selma et al. (2007) applied aqueous ozone at 5 ppm during 5 min to shredded lettuce, and achieved 1.8 log CFU reduction of

*Shigella sonnei*. Vurma et al. (2009) used ozone (5-10 ppm) in the gaseous form to decontaminate spinach leaves. Those authors obtained 1.8 log $CFU.g^{-1}$ reduction of *E. coli* O157:H7 (Vurma et al., 2009). Khadre and Yousef (2001) disinfected SS surfaces with aqueous ozone (5.9 ppm) for 1 min and achieved complete elimination of the microflora present (*B. subtilis* and *P. fluorescens*). Rosenblum et al. (2012) treated process water contaminated with *B. subtilis* with 2 ppm ozone for 10 min causing 1.56 log $CFU.mL^{-1}$ reduction. The antimicrobial effective concentrations of ozone are much lower when compared to SH. However, the corrosiveness and the low stability (difficulty to maintain a constant residual concentration) have to be considered (Simões and Simões, 2013).

### 2.3.7. Quaternary ammonium compounds

Quaternary ammonium compounds (QACs) are cationic surfactants (Ramos et al., 2013) usually used at concentrations between 200 and 400 ppm for the disinfection of food-contact surfaces (Chauret, 2014). Their mode of action is promoted through the interference with the lipid bilayer of membranes (Velázquez et al., 2009). These disinfectants have little effect on spores (Holah, 2014), but are highly effective against Gram-positive bacteria (Chaidez et al., 2007, Ohta et al., 2008, Ramos et al., 2013). The main advantages of QACs are the following: i) their stability in solution; ii) long shelf-life; iii) environmentally friendly nature; iv) safe to handle; v) non-corrosive nature (Holah, 2014); vi) odorless; vii) effective in a wide range of temperature and pH conditions (Bari and Kawamoto, 2014); and viii) able to disinfect food-contact surfaces more easily than other disinfectants (Ramos et al., 2013). However, like chlorine, QACs antimicrobial activity is affected by the presence of organic matter (Holah, 2014). Moreover, they have low activity in hard water (Bari and Kawamoto, 2014, Holah, 2014) and are not approved for direct contact with food (Ramos et al., 2013). Benzalkonium chloride, benzethonium chloride and cetylpyridinium chloride are examples of commonly used QACs (Izumi, 2014). Velázquez et al. (2009) showed that benzalkonium chloride (0.1 ppm) damaged lettuce leaves with the appearance of yellow spots on the produce after 7 days of storage. On the other hand, Park et al. (2013) proved the efficacy of this disinfectant against microorganisms (*B. cereus*, *S. aureus* and *E. coli*) isolated from fresh-cut products. Microbial growth was completely inhibited when used at 2 ppm (Park et al., 2013). Wang et al. (2001) used cetylpyridinium chloride at $5\times10^3$ ppm (much higher concentration than the one used for benzalkonium chloride) to decontaminate broccoli,

cauliflower, and radishes and obtained reductions of 3.70, 3.15 and 1.56 log CFU.$g^{-1}$ for *L. monocytogenes*, *S.* Typhimurium and *E. coli* O157:H7, respectively. Chaidez et al. (2007) used a mixture of n-alkyl dimethyl benzyl ammonium chloride sulfosuccinate dioetil at 100 ppm and urea at 200 ppm and found that *E. coli* was more resistant to the treatment with QACs than *S. aureus*.

### 2.3.8. Sodium bicarbonate

Sodium bicarbonate (SB) is currently used as food additive, has GRAS status and has a wide acceptance by the consumers and the food industry, as it is non-toxic and it does not cause damage to the fruits and vegetables (Smilanick et al., 1999). Furthermore, it has a low cost and can also be used to disinfect food-contact surfaces such as SS (Malik and Goyal, 2006). SB has been used to control green and blue molds on citrus (Smilanick et al., 1999, Palou et al., 2001). Palou et al. (2001) evaluated the effects of a treatment with SB (2.5 min at room temperature) for the control of blue mold caused by *Penicillium italicum* on citrus. A solution of $1\times10^4$ ppm was not effective, but solutions of $2\times10^4$ to $4\times10^4$ ppm reduced the blue mold by 50% (Palou et al., 2001). Smilanick et al. (1999) found that $4.2\times10^5$ and $8.3\times10^5$ ppm were the effective doses necessary to inhibit 50% and 95% of *P. digitatum* spores, respectively. Malik and Goyal (2006) used SB at a concentration of $5\times10^4$ ppm and obtained 99.22% reduction of feline calicivirus on food-contact surfaces within 1 min of exposure time. As it was described for HP, SB has to be applied in higher concentrations than SH (Smilanick et al., 1999).

### 2.3.9. Weak Organic Acids

Weak organic acids, natural or chemically synthetized, are commonly used as preservatives in the food industry (Hirshfield et al., 2003, Lianou et al., 2012). Their application is well accepted by the consumers since most of them are naturally present in foods as ingredients. Many organic acids have GRAS status and are FDA and EC (European Commission) approved. Besides their use as preservatives, they are also used as antioxidants, flavoring agents, acidulants and pH regulators (Carpenter and Broadbent, 2009). Citric, acetic and lactic acids are the most common acids applied in the food industry (Rico et al., 2007, Ölmez and Kretzschmar, 2009). Their mode of action is based on the acidification of the cytoplasm, disruption of proton motive force, osmotic stress and inhibition of macromolecule synthesis (Brul and Coote, 1999, Hirshfield et al., 2003, Carpenter and Broadbent, 2009). Furthermore, they have a quick mode of action against

an extensive range of bacteria grown under varying temperatures (Hirshfield et al., 2003, Sagong et al., 2011). Organic acids have advantages towards SH when used as disinfectants for the fresh-cut industry, as their interaction with organic molecules do not produce toxic or carcinogenic compounds (Lianou et al., 2012). Their possible disadvantage could be the change in the flavor of the product that could influence its sensorial analysis. Furthermore, when organic acids are used to disinfect fresh-produce, the wastewater may present high values of both chemical oxygen demand (COD) and biochemical oxygen demand (BOD) (Ölmez and Kretzschmar, 2009). Other pointed disadvantages are their high cost and the corrosiveness of the processing equipment that they may provoke (Sagong et al., 2011).

Citric acid is a preservative and flavoring agent usually applied in the food and pharmaceutical industries (Ölmez and Kretzschmar, 2009). Contrary to the action of the other acids, this acts as a chelating agent of metallic ions present in the medium, preventing microbial proliferation (Gurtler and Mai, 2014). Samara and Koutsoumanis (2009) used citric acid ($5\times10^3$ to $1\times10^4$ ppm) at 20 ºC for 1 to 5 min to control *L. monocytogenes* on lettuce and obtained 1 log CFU.cm$^{-2}$ reduction. Acetic acid is soluble in lipids and therefore is able to diffuse through the cytoplasmic membrane, affecting the intracellular pH of microorganisms, causing cell death (Lianou et al., 2012). Akbas and Olmez (2007) tested concentrations of $5\times10^3$ to $1\times10^4$ ppm of acetic and lactic acids (used in separate) (20 ºC for 2 to 5 min) in order to decontaminate lettuce leafs. They were able to reduce the populations of *E. coli* and *L. monocytogenes* with acetic acid by 2.2 and 1.3 log CFU.g$^{-1}$, respectively; lactic acid caused reductions of 2.8 and 2.1 log CFU.g$^{-1}$, respectively (Akbas and Olmez, 2007). The available research clearly shows that in order to have significant antimicrobial effects these organic acids have to be used in much higher concentrations than SH.

There are other organic acids that can also be used in the food industry to control microbial growth, such as peracetic acid (PA), which is usually applied as a disinfecting agent of food-contact surfaces under lower concentrations than the other mentioned organic acids (da Silva Fernandes et al., 2015). This acid combines the active oxygen characteristics of peroxide within an acetic acid molecule. It is sporicidal and very efficient due to its high oxidizing potential (Martín-Espada et al., 2014, Sudhaus et al., 2014). It is believed that PA acts by disrupting the chemiosmotic function of the cytoplasmic membrane (Kitis, 2004). In a study of Vandekinderen et al. (2009) a

treatment of fresh-cut iceberg lettuce with 120 ppm of PA reduced the native microbial load by 1.2 log CFU.g$^{-1}$ without affecting the sensorial or nutritional quality of the product. Ge et al. (2013) achieved 0.99 log CFU.g$^{-1}$ reduction of *S.* Typhimurium on lettuce by applying PA at 40 ppm for 5 min. Park et al. (2011) studied the decontaminating effects of propionic, acetic, lactic, malic, and citric acid ($1\times10^4$ ppm, 10 min) against *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* on organic fresh lettuce obtaining reductions of 0.93-1.52 (propionic), 1.13-1.74 (acetic), 1.87-2.54 (lactic), 2.32-2.98 (malic) and 1.85-2.86 (citric) log CFU.g$^{-1}$. These authors suggested that organic acids are relevant for decontamination of fresh produce.

## 2.4. Physical-based methods

### 2.4.1. Ionizing irradiation

Ionizing irradiation, such as x-rays, gamma-rays and electron beams, produces ions and electrically charged atoms and molecules. The mode of action of all these forms of ionizing radiation is similar: they act on water molecules forming free radicals that destroy or inhibit microorganisms (Ramos et al., 2013). Despite the fact that this method is quite effective in microbial growth control, FDA only approves the use of a maximum level of 1.0 kGy to decontaminate vegetables. Thus, if the produce is treated with doses higher than 1.0 kGy it cannot be designated as "fresh" (21CFR101.95, 2014). Furthermore, this physical method should better be used in combination with a chemical method, as it only reduces the microbial load to facilitate further chemical disinfection (Doona et al., 2015). The main advantages of the ionizing radiation are the very low energy requirements and the reduced heating of the food (Ramos et al., 2013). This method has not yet been adopted in the fresh produce industry mainly because: i) further research is needed to determine the necessary doses for different products (Goodburn and Wallace, 2013); ii) the consumer still have a strong negative perception of irradiated foods (Goodburn and Wallace, 2013, Ramos et al., 2013); iii) the quality of the fresh produce can be affected, especially at high doses (Ramos et al., 2013).

### 2.4.2. Membrane filtration

Membrane separation can be used to treat the process water, to avoid cross-contamination of the produce (Allende et al., 2008, Gil et al., 2009). This procedure involves the flow of water through a semipermeable membrane and the consequent retention of the

undesired contaminants on the membrane. Microfiltration (MF), ultrafiltration (UF), nanofiltration (NF) and reverse osmosis (RO) are membrane unit operations that can be applied in the food industry to treat the process water (Cassano and Basile, 2013).

MF is a dead-end filtration where the feed flows vertical to the membrane and all the solids become retained on the membrane. In MF the membrane pore size is between 0.05 and 10 µm (Berk, 2009, Salehi, 2014), the molecular weight cut off (MWCO) is 200 kDa (Singh, 2015) and the pressure used is below 0.2 MPa (Salehi, 2014). This membrane process is usually applied for the retention of microorganisms (Salehi, 2014). UF membranes have a pore size of 0.001-0.05 µm, MWCO between 1 and 300 kDa (Singh, 2015) and a pressure of 0.2-0.5 MPa. UF is usually used to separate proteins and other high molecular weight organic compounds (Salehi, 2014, Singh, 2015). Regarding NF, the membrane pore size is lower than 2 nm (Salehi, 2014), the MWCO is between 100 and 1000 Da (Singh, 2015) and the pressure is of 0.5-1.5 MPa (Salehi, 2014). Normally NF is used for water softening and the removal of salts (Singh, 2015). In RO the membrane has a pore size of 0.6 nm (Singh, 2015), with MWCO of 100 Da (Salehi, 2014) and the pressure used is between 1.5 and 10 MPa (Berk, 2009, Salehi, 2014, Singh, 2015). The main disadvantage of these methods is the high investment costs associated to the implementation of a membrane separation unit (Casani et al., 2005). Moreover, the costs (acquisition, energy and maintenance) of membrane technology and the limited life span, due to the high pressure drop caused by biofilms, reduces their wide use in the food industry (Melo and Flemming, 2010).

### 2.4.3. Steam jet-injection

Steam jet-injection is a heat treatment that destroys microorganisms and inactivates enzymes that might be responsible for produce spoilage (Rico et al., 2008a). The heat time exposure is usually short (≈10 seconds). Consequently, the impairment of organoleptic properties is reduced (Martín-Diana et al., 2007). However, the heat exposure still promotes the loss or reduction of the bioavailability of some nutrients (Rico et al., 2008a). Moreover, the high power consumption associated to the steam jet formation and also the high temperature of the effluents generated have to be considered (Martín-Diana et al., 2007, Rico et al., 2008a). Rico et al. (2008a) reported that 10 seconds was the ideal time to obtain a satisfactory mesophilic load reduction for fresh-cut lettuce. In fact, the authors observed a significant loss of vitamin C and carotenoids for longer treatments. Martín-Diana et al. (2007) concluded that the mesophilic load reduction of

fresh-cut lettuce was the same when using steam jet-injection and chlorine at 120 ppm.

### 2.4.4. Temperature

Control of temperature is a key point in microbial growth control. Either refrigeration or heating of water can be applied to control or reduce microbial load, respectively. Furthermore, the air temperature can also be reduced to delay microbial proliferation. However, as previously stated for irradiation, this physical method should be used as complement, as it is not usually effective alone to ensure the desired microbiological safety of product. Low temperatures can delay food spoilage, but also mask the latent state of the pathogens (Parish et al., 2003). For a product to be considered fresh, the "high" temperatures used have a defined threshold (≈40 – 60 ºC, 1-5 min) (Parish et al., 2003, 21CFR101.95, 2014, Fallik, 2014). Moreover, the high temperatures can induce damages of the produce tissues favoring microbial entrance and consequent spoilage (Parish et al., 2003).

### 2.4.5. Ultrasounds

Ultrasounds (US) are sonic waves at high amplitude, above human-hearing threshold (>20 kHz) (Otto et al., 2011, Paniwnyk, 2014) that form cavitation bubbles (Seymour et al., 2002). These bubbles collapse generating the mechanical energy responsible for the disinfecting action (detachment) and the chemical energy responsible for the free radicals formation (destruction) (Seymour et al., 2002, Bermúdez-Aguirre et al., 2011, Sagong et al., 2011), increasing the permeability of cell membranes (Bilek and Turantaş, 2013). By this collapse, hot spots are formed (high temperatures and pressure) and free radicals are released, causing DNA modifications in the cells (São José et al., 2014). In the food industry, US are used at low frequencies, in the range of 20-100 kHz (Sagong et al., 2011, Paniwnyk, 2014), and require the presence of a fluid for transmission. The high-intensity treatments necessary to inactivate the microorganisms can be a drawback as these can affect the organoleptic properties of the produce (Seymour et al., 2002). Microbial resistance to US varies according to the cell shape (coccus are more resistant), size (smaller cells are more resistant), Gram nature (Gram-positive bacteria are more tolerant) and cellular metabolism (aerobic microorganisms are more resistant) (Chemat et al., 2011, Paniwnyk, 2014).

Comparing this technique with chlorine, the main advantages are the safety associated with the sound waves and also the fact that it is environmentally friendly (Kentish and

Ashokkumar, 2011). The UK Health Protection Agency (HPA) recommends an exposure limit for the general public to airborne ultrasound sound pressure levels (SPL) of 70 dB (at 20 kHz), and 100 dB (at 25 kHz and above) (AGNIR, 2010). This method is effective at an optimum temperature of 60 °C, which is definitely a disadvantage when working with fresh produce (high temperatures can change the food properties). Seymour et al. (2002) used water at temperatures of 5 °C or 20 °C in the treatment of iceberg lettuce with US (5 min) and they concluded that both temperatures had no influence on the organoleptic properties of the fresh food. Therefore, the highest temperature is more favorable as the effects of US on iceberg lettuce decontamination are increased. The water hardness and the amount of dissolved gases have to be taken into account, due to the fact that their variability can reduce the cavitation process. Given that US should be applied for a short period of time, they do not affect the aspect of the produce (Sagong et al., 2011). The antimicrobial efficacy of US is rather limited when these are applied alone. Therefore, US can also be used in combination with other disinfectants and decontamination technologies, such as chlorine, improving the antimicrobial efficacy (São José et al., 2014).

This physical method has been extensively studied in the food industry for produce decontamination and water disinfection. Seymour et al. (2002) reported 1.5 log $CFU.g^{-1}$ reduction of *S.* Typhimurium on cut iceberg lettuce at 32-40 kHz for 10 min. These authors tested the effects of different frequencies (25, 32 and 70 kHz) and found no statistical significant differences (Seymour et al., 2002). Birmpa et al. (2013) achieved 2.30, 5.72 and 1.88 log $CFU.g^{-1}$ reduction of *E. coli*, *S.* Enteritidis, *L. innocua*, respectively, on lettuce at 37 kHz for 30 min. Kim et al. (2006) obtained a lower log $CFU.g^{-1}$ reduction (1.08) of *E. coli* O157:H7 on broccoli seeds using the same treatment time and a higher frequency (40 kHz, at 23 ºC for 30 min). The disinfection of the washing water is also important and Elizaquível et al. (2012) found a 4.4 log $CFU.mL^{-1}$ reduction of *E. coli* O157:H7 in the washing water using US at 20 kHz for 53 min, which is obviously a high exposure time and therefore not appropriate for the fresh-cut industry.

### 2.4.6. Ultraviolet light

Ultraviolet (UV) light is an electromagnetic radiation with wavelengths ranging between 100 and 400 nm. It is subdivided in four groups: UV-A, UV-B, UV-C and vacuum UV (Gray, 2014). The UV-C (190-280 nm) light (Artés et al., 2009) is used as antimicrobial as this induces DNA damages, leading to cell death (Birmpa et al., 2013). However, at

lower doses the microorganisms can remain alive, due to their DNA repair mechanisms (Shama, 2014). Insomuch the appropriate precautionary measures are taken, it is a non-toxic, safe and environmentally friendly treatment (Otto et al., 2011), however, its prolonged use can alter the organoleptic properties of the food (Demirci and Krishnamurthy, 2010). UV light can be used for disinfection by either applying a continuous mode (UV lamps) (Gray, 2014), or pulsed UV light (Condón et al., 2014). UV lamps have a tube with a gas (xenon or krypton), mercury and also have an electrode at each side of the tube. When electrical current is passed, the mercury atoms become excited and UV light is produced when the atoms return to their basal state (Gray, 2014). The advantages of UV lamps are their high efficiency (depending on the dose and exposure time) and the reduced process times (Birmpa et al., 2013). When compared to UV lamps, the mode in pulsed UV light is not continuous (the pulse rate is 1 to 20 pulses per second), therefore the energy is multiplied (100 to 1100 nm) being more efficient (Demirci and Krishnamurthy, 2010). Another advantage is the fact that pulsed UV light can be a mercury free alternative. The main drawback is the temperature increase, which can damage the produce (Demirci and Krishnamurthy, 2010, Condón et al., 2014).

The method of disinfection by using UV light is usually applied for wastewater treatment (Hunter and Townsend, 2010), while there are few applications in the food industry. As examples, Birmpa et al. (2013) used UV (254 nm) to reduce the microbial load on lettuce. The treatment reduced 1.75, 1.27, 1.39 and 1.21 log $CFU.g^{-1}$ the populations of *E. coli*, *L. innocua*, *S.* Enteritidis and *S. aureus*, respectively (Birmpa et al., 2013). Bermúdez-Aguirre and Barbosa-Cánovas (2013) used UV light (253.7 nm) to decontaminate lettuce, obtaining 1.7 log $CFU.g^{-1}$ inactivation of *E. coli* within 1 h of treatment, which is a long exposure time for the log CFU reduction observed. Ge et al. (2013) achieved 2.28 log $CFU.g^{-1}$ reduction of *S.* Typhimurium on lettuce by applying UV-C treatment for 5 min with an irradiation fluency of 450 $mJ.cm^{-2}$.

## 2.5. Combination of disinfection methods

Most of the biological, chemical and physical methods which were previously described have reduced effectiveness in microbial growth control when applied alone. Therefore, these methods have to be combined in order to increase their antimicrobial efficacy. Moreover, when combined with chlorine they will help reduce the use of chlorine to achieve the desired antimicrobial effect. Combinations of physical-chemical (Gabriel,

2015), chemical-chemical (Singh et al., 2002b), chemical-biological (Arevalos-Sánchez et al., 2012) and biological-biological (Lequette et al., 2010) methods have already been successfully described. The main aim of these combinations is to achieve a more effective disinfection process. In fact, the combination of diverse methods allows a wider antimicrobial action (Goodburn and Wallace, 2013).

Ionizing irradiation is a method to decontaminate fresh produce that is mandatorily combined with a chemical method. Foley et al. (2002) combined this method with chlorine and proved that applying 0.55 kGy of gamma-rays together with 200 ppm of chlorine on shredded iceberg lettuce were able to reduce the population of *E. coli* O157:H7 by 5.4 log CFU.g$^{-1}$. However, they used a high concentration of chlorine which is not the purpose of combining the two methods. Huang and Chen (2011) combined a physical process (heating at 50 ºC) with HP ($2\times10^4$ ppm) to decontaminate baby spinach. When the physical-chemical combination was applied, the reduction of *E. coli* O157:H7 was 2.2 log CFU.g$^{-1}$, and when HP was used alone, the reduction was significantly lower (Huang and Chen, 2011). Delaquis et al. (2004) also combined high temperature with chlorine at 100 ppm. The authors found that the heating of the fresh-cut iceberg lettuce at 50 ºC for 1 min resulted in 1.5 log CFU.g$^{-1}$ reductions of the total microbial population, while the combination with chlorine caused an additional 0.5 log CFU.g$^{-1}$ reduction (Delaquis et al., 2004). Nevertheless, the use of high temperatures can affect the organoleptic properties of the produce (Parish et al., 2003). Seymour et al. (2002) combined US (32-40 kHz) with 25 ppm chlorine in a 10 min treatment to eliminate *S.* Typhimurium from fresh-cut lettuce. These authors achieved 2.7 log CFU.g$^{-1}$ reduction, which was higher than the reduction obtained with chlorine (1.7 log CFU.g$^{-1}$) or US (1.5 log CFU.g$^{-1}$) alone. Sagong et al. (2011) reported the combination of US (40 kHz) with lactic acid ($2\times10^4$ ppm) to decontaminate organic lettuces for 5 min at 20 ºC. They observed 2.75, 2.71 and 2.50 log CFU.g$^{-1}$ reductions of *E. coli* O157:H7, *S.* Typhimurium and *L. monocytogenes*, respectively (Sagong et al., 2011). The combination of UV (15 W UV-C lamp) and US (frequencies switched between 28, 45 and 100 kHz at 1 millisecond time intervals) was also assessed by Gabriel (2015). This combination decreased the time necessary to obtain the same effect (5 log CFU.mL$^{-1}$ reduction of *E. coli*) compared to when both technologies were applied alone (Gabriel, 2015). Rico et al. (2006) combined calcium lactate ($1.5\times10^4$ ppm, at 50 ºC) with ozone (1 ppm) to extend the shelf life of lettuce, observing a reduced enzymatic browning. The efficiency of CD (20-40 ppm) was

also improved in combination with US (170 kHz), reducing *Salmonella* and *E. coli* O157:H7 on lettuce by 2.6 and 1.8 log CFU.g$^{-1}$ (Huang et al., 2006). Ibrahim et al. (2008) combined 5 ppm ozone with 40 ppm CD for 5 min to decontaminate turnip greens and the total bacterial count was reduced by 2.17 log CFU.g$^{-1}$.

The combination of copper with lactic acid was considered antimicrobial synergistic by Ibrahim et al. (2008). Gyawali et al. (2011) observed 3.93 log CFU.cm$^{-2}$ reduction of *E. coli* O157:H7 on lettuce surface combining 40 ppm copper and $2\times10^3$ ppm lactic acid. Rahman et al. (2010) studied cabbage decontamination combining AlEOW (100 ppm) with citric acid ($1\times10^4$ ppm) at 50 ºC, reducing *L. monocytogenes* and *E. coli* O157:H7 by 3.99 and 4.19 log CFU.g$^{-1}$, respectively. Citric acid ($1\times10^4$ ppm) was combined with ozonated water (3 ppm) for 1 min to decontaminate lettuce (Yuk et al., 2006). When 5 ppm ozone was used for 5 min it caused 1.09 and 0.94 log CFU.g$^{-1}$ reduction of the populations of *E. coli* O157:H7 and *L. monocytogenes*, respectively. However, when ozone was combined with citric acid higher reductions were observed: 2.31 and 1.84 log CFU.g$^{-1}$ for *E. coli* O157:H7 and *L. monocytogenes*, respectively (Yuk et al., 2006). Combining sodium bicarbonate with HP proved to be more efficient than using sodium bicarbonate alone. Malik and Goyal (2006) reduced 99.68% the population of feline calcivirus by combining $2\times10^4$ ppm of sodium bicarbonate with $2\times10^4$ ppm of HP for 10 min. Van Haute et al. (2015a) combined PA (20 ppm) and lactic acid (4000 ppm) to treat the wash water of fresh-cut leafy vegetables. The authors proved that the use of this treatment was better than the use of chlorine, however, higher disinfectant concentrations are needed because the inactivation of *E. coli* O157 is slower, when compared to the chlorine kinetics. Enzymes have also been used in combination with US in order to remove *E. coli* biofilms from SS surfaces (Oulahal-Lagsir et al., 2003). When US (40 kHz) were used alone, 30% of biofilm mass removal was achieved. However, when US were used in combination with enzymes (trypsin) biofilm removal reached 76% (Oulahal-Lagsir et al., 2003). The combination of UV and ozone was also applied by Selma et al. (2008) achieving 6.6 log CFU.mL$^{-1}$ microbial reduction in escarole wash water. Ge et al. (2013) studied the combination of UV-C with chlorine and UV-C with PA. When they combined UV-C (irradiation fluency of 900 mJ.cm$^{-2}$, 10 min) with chlorine (200 ppm, 10 min) 2.40 log CFU.g$^{-1}$ reduction of *S.* Typhimurium on lettuce was achieved; when combining UV-C (irradiation fluency of 900 mJ.cm$^{-2}$, 10 min) with PA (80 ppm, 10 min) 2.52 log CFU.g$^{-1}$ was obtained. The treatments applied alone caused 2.29, 0.99 and 0.95

log CFU.$g^{-1}$ reduction for UV-C (irradiation fluency of 900 mJ.$cm^{-2}$, 10 min), chlorine and PA, respectively (Ge et al., 2013). From all the previous examples, it becomes rather clear that the combination of methods is a promising effort to replace and/or reduce the use of chlorine.

Despite the advantages of the alternative methods, which were here described, much research is still needed for the discovery and development of new strategies and for their effective use at industrial scale.

# 3

## MATERIALS AND METHODS

In this section the methodology used in this work will be described.

## 3.1. Microorganisms

*E. coli* CECT 434 was selected as reference strain since it has already been used as model microorganism for antimicrobial tests (Borges et al., 2012, Abreu et al., 2014). Bacteria from a MPV industry were isolated and were also studied. *S. aureus* CECT 976 was used as a control (as well as *E. coli* CECT 434) for the antibiotics tests performed in section 3.4. For quorum sensing (QS) studies *Chromobacterium violaceum* ATCC 12472 was used to determine QS inhibition (QSI) activity, since this bacterium produces and responds to the autoinducers (AI) C6-AHL (N-acyl homoserine lactone) and C4-AHL. To detect the type of molecule responsible for QSI a bioassay for AHL production was performed according to McLean et al. (2004). For this two bacteria were used: *C. violaceum* CV026 and *Agrobacterium tumefaciens* A136. These two bacteria lack AHL synthase genes, but respond to exogenous active signal molecules: *C. violaceum* CV026 is sensitive to C4-AHL and C6-AHL and *A. tumefaciens* A136 is sensitive to AHL from C6-C14. The strains *C. violaceum* ATCC 31532 and *A. tumefaciens* KYC6 were used as positive controls.

All microbial strains were stored at -80 ± 2 °C in cryovials with doubly concentrated medium and 30% ($v.v^{-1}$) glycerol (Panreac, Spain). They were subcultured in the respective solid medium (medium with agar) before testing. All media were prepared according to the manufacturer instruction and sterilized in an autoclave (Uniclave 88, AJC, Portugal) at 121 °C for 20 min.

### 3.1.1. MPV bacteria isolation

The sampling was performed in the spring (May) in a MPV industry in Portugal processing 2500 tons of salad leaves per year. The scheme of the processing line studied can be observed in Figure 3.1. The washing room comprises the washing and sanitizing tank. The high care area englobes the optical sorting and packaging. Both rooms are at 5 ºC and are separated from a hole in the wall where the conveyor belt goes through. The washing tank was filled with tap water and allowed the removal of dirt and debris. The

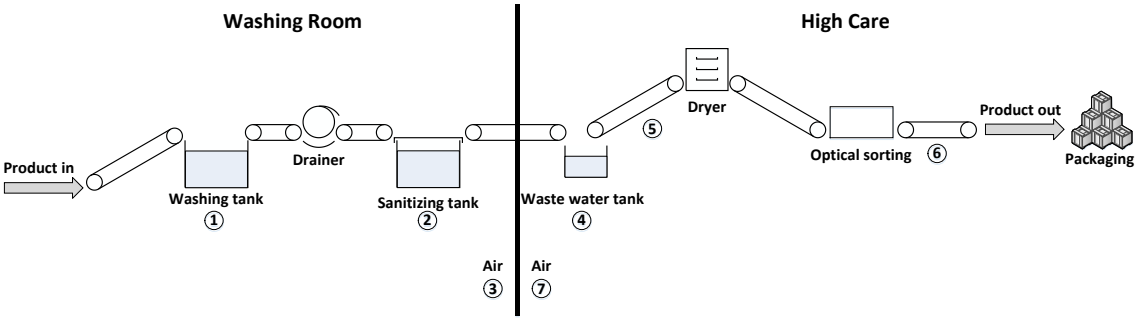


**Figure 3.1.** Schematic representation of the MPV plant and the steps involving the produce processing. The numbers correspond to the sampling points: ① Washing tank; ② Sanitizing tank; ③ Air; ④ Waste water tank; ⑤ Conveyor belt before dryer; ⑥ Conveyor belt after optical sorting; ⑦ Air.

sanitizing tank had tap water and SH (70 – 90 ppm) allowing the microbial load reduction. The water was changed when the facilities (equipment and tanks) were sanitized (after operating continuously for 18 h) with Tego 2000® (JohnsonDiversey, United Kingdom) for 2 h. Tego 2000® is a trademark disinfectant with full composition unknown. The only information known from the security sheet is that it has amines, n-C10-16-alkyltrimethylenedi- and reaction products with chloroacetic acid and acetic acid (JohnsonDiversey, 2004).

The samples were collected from food contact surfaces (before the sanitization procedure - during processing - and after the sanitization procedure with Tego 2000®), from baby green Batavia leaves (during processing) and from the surrounding air (during the sanitization procedure with Tego 2000®). Both washing room and high care area of the MPV industry were sampled (Figure 3.1).

The sampling from the food-contact surfaces was done by cotton swabbing, already proposed as a reliable sampling method by Verran et al. (2010). The swabbing was done 5 cm above and below the line of water (on a surface area of 10 cm) and the collection swab was placed in 50 mL tubes with 25 mL of medium in each tube. The procedure was repeated three times in order to place a cotton swab in each medium used (Man, Rogosa and Sharpe broth (MRS; Merck, Germany), tryptic soy broth (TSB; Merck, Germany) and buffered peptone water (BPW; Merck, Germany)). To place the cotton swab in the tubes with medium they were opened in sterile conditions using a fire flame. For all the samples collected in the 50 mL tubes it was applied the spread-plate method. In this method 100 μL of each sample was spread on agar plates containing the same medium of the tube (except for the tubes with buffered peptone water, where it was used plate count

agar (PCA; Oxoid, England). This procedure was done in duplicate. The plates were grown at 30 °C during 72 hours. Baby green Batavia leaves (6 cm) were placed in 50 mL tubes with 25 mL of each medium in each tube for the three different media (three leaves in each tube). Samples of 100 µL were retrieved from each tube with different medium and were spread on agar plates of MRS (Merck, Germany), TSB (Merck, Germany) and PCA (Oxoid, England). This procedure was done in duplicate. The plates were grown at 30 °C during 72 hours. The air sampling was performed based on the microbial fallout on to PCA (Oxoid, England) plates (passive air sampling) according to Pasquarella et al. (2000). The plates were dispersed over the two areas (6 plates in the washing room and 3 plates in the high care) and left opened for 2 h. The plates from air sampling were left to incubate for 72 h at 30 °C.

Each colony obtained from the sampling was then streaked to a new plate from the same medium as the one used in the first steps of isolation (except for BPW where PCA was used). The isolated bacteria were grown at 30 ºC.

### 3.1.2. MPV bacteria identification

To identify the bacteria, two to three colonies were resuspended in 65 µL of phosphate buffer saline (PBS). The solution was homogenized with vortex. Then five drops of 10 µL of the cells suspension were placed on FTA cards (Whatman, UK) and left to dry at room temperature (Figure 3.2). These FTA cards have a patented formula that lyses the cells'


**Figure 3.2.** FTA cards with the isolates.

membranes and denatures proteins. Once the immediate reaction occurs, nucleic acids are protected from external sources, such as UV light (Rajendram et al., 2006). The cards were sent to StabVida (Portugal) where the identification was performed (a small disc from the cards was washed and used in PCR analysis). The diverse isolates were identified by 16S rRNA sequencing using four primers 27F, 518F, 800R e 1492R (Nuobariene et al., 2015). The DNA sequences were aligned with ClustalW and the phylogenic tree was constructed with MEGA 6 (Tamura et al., 2013) using the Neighbor-Joining method (Saitou and Nei, 1987). The tree was analyzed with bootstrap test (1000 replicates) (Felsenstein, 1985). The evolutionary distances were computed using the p-distance method (Nei and Kumar, 2000).

### 3.2. Production of virulence factors

The virulence factors tested were siderophores, proteases and gelatinases. The bacteria (all the isolates and *E. coli* CECT 434) were obtained from overnight cultures grown in 50 mL tubes with 20 mL of TSB (Merck, Germany), incubated at 30 ºC and 120 rpm (CERTOMAT® BS-1, Sartorius AG, Germany). An optical density ($O.D._{600nm}$) of 0.2 was obtained in a test tube with saline solution (NaCl) at 8.5 $g.L^{-1}$. At least three independent experiments were performed for all the tests.

To detect siderophores production, chrome azurol S (CAS) agar plates were prepared according to the method used by Schwyn and Neilands (1987). Ten µL of the microbial suspension were placed in each 90 mm plate of CAS agar plates (in three different positions). The plates were incubated at 30 °C, for 48 h. After the incubation time, an orange halo was measured, indicating the production of siderophores (Schwyn and Neilands, 1987, Neilands, 1995, Milagres et al., 1999).

For proteases production, 10 µL of the microbial suspension (in three different positions) were placed in each 90 mm plate of PCA (Oxoid, England) with 1 $g.L^{-1}$ skim milk powder (Merck, Germany). The plates were incubated at 30 °C, for 72 h. After the incubation time, the plates were flooded with 1.00 $mol.L^{-1}$ hydrochloric acid (Fisher Scientific, UK). The clearance zones formed indicated protease positive strains (Dogan and Boor, 2003).

Regarding gelatinases production, 10 µL of the microbial suspension (in three different positions) were placed in each 90 mm plate of gelatin agar plate (5 $g.L^{-1}$ peptone (Oxoide, UK), 3 $g.L^{-1}$ yeast extract (Merck, Germany), 30 $g.L^{-1}$ gelatin (Oxoid, UK), 15 $g.L^{-1}$ agar (Oxoid, UK), at pH 7). The plates were incubated at 30 ºC, for 48 h. After the incubation

period, the plates were flooded with 2.84 mol.$L^{-1}$ ammonium sulfate (Panreac, Spain). If the bacteria produced gelatinase, a transparent halo around cells would appear (gelatin precipitates) (Lopes et al., 2006).

## 3.3. Quorum sensing inhibition

The bacteria (isolates and *E. coli* CECT 434) were streaked on Luria-Bertani (LB; Liofilchem, Italy) agar plates and grown overnight at 30 °C. *C. violaceum* ATCC 12472 was used as the indicator microorganism and was grown at 30 °C overnight in LB broth (LBB). After overnight growth the isolated bacteria were overlaid with LB soft agar (10 g.$L^{-1}$) containing *C. violaceum* ATCC 12472 in an $O.D._{600nm}$ of 0.1. The plates were incubated overnight at 30 ºC. A positive quorum sensing inhibitor was indicated by the lack of pigmentation of *C. violaceum* ATCC 12472 around the bacteria (McLean et al., 2004). At least three independent experiments were performed.

### 3.3.1. N-acyl homoserine lactones (AHL) production

To detect the type of molecule responsible for QSI a bioassay for AHL production was performed according to McLean et al. (2004). The bacteria demonstrating ability to inhibit QS, were streaked on LB agar plates and grown overnight at 30 °C. The indicator microorganisms (*C. violaceum* CV026 and *Agrobacterium tumefaciens* A136) were grown overnight in LBB (for *A. tumefaciens* A136 the broth was supplemented with 50 µg.$mL^{-1}$ spectinomycin (Sigma Aldrich, Portugal) and 4.5 µg.$mL^{-1}$ tetracycline (Sigma Aldrich, Portugal)) and incubated at 30 °C and 120 rpm (CERTOMAT® BS-1, Sartorius AG, Germany). *C. violaceum* CV026 is sensitive to C4-HSL (*N*-butanoyl homoserine lactone) and C6-HSL (*N*-hexanoyl homoserine lactone) and *A. tumefaciens* A136 is sensitive to AHL from C6-C14. After overnight growth the isolated bacteria were overlaid with LB soft agar (10 g.$L^{-1}$) containing *C. violaceum* CV026 and *A. tumefaciens* A136 in an $O.D._{600nm}$ of 0.1 and the plates were incubated again (24 h). After incubation, 20 mg.$mL^{-1}$ X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; Fisher Scientific, USA) prepared in dimethylformamide was added to the plates overlaid with *A. tumefaciens* A136. A positive test was indicated by a blue coloration (X-Gal hydrolysis) in *A. tumefaciens* A136. In the tests with *C. violaceum* CV026 a positive test was indicated by a purple pigmentation around the bacteria (McLean et al., 2004). The

strains *C. violaceum* ATCC 31532 and *A. tumefaciens* KYC6 were used as positive controls. At least three independent experiments were performed.

### 3.4. Antibiotic susceptibility

Antibiotics from different classes were selected for this study (Davies, 2013): tetracycline (Sigma-Aldrich, China) 30 µg/disc, erythromycin (AppliChem, Germany) 15 µg/disc, levofloxacin (Alfa Aesar, USA) 5 µg/disc, ampicillin (AppliChem, Germany) 10 µg/disc, and ciprofloxacin (Fluka, China) 5 µg/disc. The Clinical & Laboratory Standards Institute (CLSI) guidelines (CLSI, 2005) do not provide information for the isolated microorganisms. Therefore, *E. coli* CECT 434 and *S. aureus* CECT 976 were used as controls and the antibiotics were selected according to these bacteria. Also the antibiotics were chosen for their different classes classification.

All the bacteria were obtained from overnight cultures grown in 50 mL tubes with 20 mL of Mueller–Hinton broth (MHB) (Merck, Germany), incubated at 30 ºC and 120 rpm (CERTOMAT® BS-1, Sartorius AG, Germany). The disc diffusion method was used to test the selected antibiotics against the isolated microorganisms. Bacterial suspensions were adjusted to an $O.D._{600nm}$ of 0.1 and using a sterilized cotton swab they were seeded on MH agar (MHA) plates. Then 6 mm sterile discs (Prat Dumas, France) were placed on the same MHA plates, as well as the tested antibiotics. After incubation (30 °C for 24 h) the inhibition zone diameter was recorded. According to CLSI (2005) the isolated bacteria were considered resistant (R), intermediate (I) or susceptible (S) for each antibiotic (Table 3.1). At least three independent experiments were performed for each condition tested.

### 3.5. Aggregation

After an overnight growth in 50 mL tubes with 20 mL of MHB (Merck, Germany), incubated at 30 ºC and 120 rpm (CERTOMAT® BS-1, Sartorius AG, Germany), the isolates cell suspensions were centrifuged at 4000 g for 20 min. Afterwards they were resuspended in 100 mM potassium chloride (VWR, Belgium) and washed two times in 100 mM potassium chloride. After the washing steps the cells were resuspended in 100 mM potassium chloride until reaching an $O.D._{600\ nm}$ of 1.5 (Ledder et al., 2008). To determine autoaggregation a volume of 3 mL (for each microbial isolate) was placed in a glass test tube at room temperature and the initial $O.D._{600nm}$ was measured. After 1 hour

at room temperature the $O.D._{600nm}$ was measured again (Ledder et al., 2008). The aggregation index was calculated according to equation 3.1:

$$\text{Aggregation index} = \left[\frac{(\text{initial O. D.}_{600} - \text{O. D.}_{600}\ \text{after 1h})}{\text{initial O. D.}_{600}}\right] \times 100 \qquad (3.1)$$

To determine visual autoaggregation the same procedure was used (a volume of 3 mL, for each microbial isolate, was placed in a glass test tube at room temperature). Then the tube was vortexed for 10 s and rolled for 30 s. The differences were observed at different times (0, 2, 24 and 48 h) (Simões et al., 2008). The aggregation index classification was according to Cisar et al. (1979): 0 - Suspensions with no evidence of aggregation or coaggregation; +1 - Detectable but finely dispersed aggregates; +2 - Aggregates that formed immediately, but remained suspended in a turbid background; +3 - Aggregates that settled rapidly but with a supernatant that remained slightly cloudy; +4 - Rapid and complete settling of large aggregates leaving a water-clear supernatant.

To study coaggregation only microorganisms with autoaggregation ability were selected and combined according to the original isolation procedure (combined if they were isolated from the same place). An equal volume of the suspensions (1.5 mL for each microorganism) was placed in the glass test tube and the initial $O.D._{600nm}$ was measured. The same procedure and classification used for autoaggregation studies was applied.

Table 3.1. Classification used for the antibiotics according to the diameter of the inhibition zone and if they are applied against Gram-positive (*S. aureus* ATCC® 25923) or Gram-negative (*E. coli* ATCC®25922) bacteria (CLSI, 2005)

| **Antibiotic** | **Class** | **Gram** | **Diameter of inhibition zone (cm)** | | |
|---|---|---|---|---|---|
| | | | **R** | **I** | **S** |
| Tetracycline | Tetracyclines | + | ≤ 1.4 | 1.5 – 1.8 | ≥ 1.9 |
| | | - | ≤ 1.4 | 1.5 – 1.8 | ≥ 1.9 |
| Erythromycin | Macrolides | + | ≤ 1.3 | 1.4 – 2.2 | ≥ 2.3 |
| | | - | ≤ 1.3* | 1.4 – 2.2* | ≥ 2.3* |
| Ampicillin | β-Lactams | + | ≤ 2.8 | - | ≥ 2.9 |
| | | - | ≤ 1.3 | 1.4 – 1.6 | ≥ 1.7 |
| Levofloxacin | Quinolone | + | ≤ 1.5 | 1.6 – 1.8 | ≥ 1.9 |
| | | - | ≤ 1.3 | 1.5 – 1.6 | ≥ 1.7 |
| Ciprofloxacin | Quinolone | + | ≤ 1.5 | 1.6 – 2.0 | ≥ 2.1 |
| | | - | ≤ 1.5 | 1.6 – 2.0 | ≥ 2.1 |

* Erythromycin is not described for *E. coli* ATCC®25922, so the values used for Gram-negative bacteria were the same used for *S. aureus* ATCC® 25923

## 3.6. Stomach environment survival

The gastric environment was simulated according to Abadía-García et al. (2013) and Guerra et al. (2012). Firstly the isolates, from overnight cultures grown in 50 mL tubes with 20 mL of TSB (Merck, Germany), incubated at 30 ºC and 120 rpm (CERTOMAT® BS-1, Sartorius AG, Germany), were added ($2\times10^9$ cells.mL$^{-1}$) to an artificial saliva medium (6.2 g.L$^{-1}$ NaCl, 2.2 g.L$^{-1}$ KCl, 0.22 g.L$^{-1}$ $CaCl_2$ and 1.2 g.L$^{-1}$ $NaHCO_3$) (Abadía-García et al., 2013) at pH 7 and 37 ºC for 1 min (Guerra et al., 2012). The volume used for the artificial saliva was in a 1:8 ratio (saliva:stomach). After this step the solution was added to a 2 L reactor stirred with a magnetic anchor to allow complete mixture. The reactor contained TSB (Merck, Germany), pepsin from porcine gastric mucosa (Panreac AppliChem, Spain) (3 g.L$^{-1}$) and the pH was adjusted to 2 with HCl (37%). The reactor was maintained at 37 ºC for 3 hours (Guerra et al., 2012, Abadía-García et al., 2013). Samples were collected after these 3 hours and the necessary dilutions were performed to enumerate the CFU using the motion drop method (Reed and Reed, 1948) on TSA plates. The plates were incubated at 30 ºC during 24 hours. At least three independent experiments were performed.

## 3.7. Biofilm formation using 96-well PS plates

### 3.7.1. Crystal violet (CV) staining

Bacteria were obtained from overnight cultures incubated at 30 ºC and 120 rpm (CERTOMAT® BS-1, Sartorius AG, Germany) and grown in 50 mL tubes with 20 mL of medium. For all isolates, TSB was the medium used. For comparison of *E. coli* CECT 434 with a food isolate (*P. oryzihabitans*) a specific medium (5.5 g.L$^{-1}$ glucose, 2.5 g.L$^{-1}$ peptone, 1.25 g.L$^{-1}$ yeast extract, 1.88 g.L$^{-1}$ monopotassium phosphate and 2.6 g.L$^{-1}$ sodium phosphate dibasic) was tested. Additionally, in the development of a new artificial lettuce medium (section 3.13) biofilm formation was quantified for the new medium and for MHB (Merck, Germany).

An $O.D._{600nm}$ of 0.1 was achieved and the cells were placed in 96-well flat-bottomed PS tissue culture plates with a lid (Orange Scientific, Belgium) using a total volume of 200 µL (Borges et al., 2012). After an incubation period (30 ºC and 120 rpm (CERTOMAT® BS-1, Sartorius AG, Germany)) of 24 h the biofilm mass was quantified by CV staining, according to Stepanović et al. (2000). The absorbance (570 nm) values were measured in

a Synergy™ HT 96-well microplate reader (Biotek Instruments, Inc., Colmar, France). The bacteria were classified in terms of biofilm production ability, according to Stepanović et al. (2000), as non-biofilm producers (0 if O.D. ≤ $O.D._C$), weak biofilm producers (+ if $O.D._C$ < O.D. ≤ 2 × $O.D._C$), moderate biofilm producers (+ + if 2 × $O.D._C$ < O.D. ≤ 4 × $O.D._C$) and strong biofilm producers (+ + + if O.D. > 4 × $O.D._C$). $O.D._C$ corresponds to the OD cut-off value (average of the O.D. of the negative control + 3 × standard deviation of the negative control). At least three independent experiments were performed.

### 3.7.2. Colony forming units (CFU)

Bacteria (*E. coli* CECT 434 and a specific food isolate (*P. oryzihabitans*)) were obtained from overnight cultures incubated at 30 ºC and 120 rpm (CERTOMAT® BS-1, Sartorius AG, Germany) and grown in 50 mL tubes with 20 mL of medium. In the development of a new artificial lettuce medium (section 3.13) biofilm formation was quantified for the new medium and for MHB.

An $O.D._{600nm}$ of 0.04 was obtained and the cells were placed in 96-well flat-bottomed PS tissue culture plates with a lid (Orange Scientific, Belgium) using a total volume of 200 µL (Borges et al., 2012). The plates were incubated at 30 ºC and 120 rpm (CERTOMAT® BS-1, Sartorius AG, Germany) during 24 h. The wells were washed with 8.5 $g.L^{-1}$ NaCl that was discarded. Then 8.5 $g.L^{-1}$ NaCl (250 µL) was added to the wells 4 times and each time the wells were scraped and the volume was collected. To determine the CFU the motion drop method was used on PCA plates and the necessary dilutions were performed in 8.5 $g.L^{-1}$ NaCl. These plates were incubated overnight, at 30 ºC. At least three independent experiments were performed.

## 3.8. Bacterial adhesion and biofilm formation on coupons

Bacterial adhesion on coupons was only performed for *E. coli* CECT 434. Biofilm formation on coupons was performed for all the isolates and *E. coli* CECT 434. Coupons of SS (AISI 316) and PS (dimensions of 1.0 × 0.9 × 0.1 cm) were used. The PS coupons were only used on *E. coli* CECT 434 tests. The coupons were placed in 48-wells flat-bottomed PS tissue culture plates (Thermo Fisher Scientific, Korea) using a total volume of 1000 µL with an initial $O.D._{600nm}$ of 0.1 (on MHB (Merck, Germany)). The plates were

incubated for 2 hours and 24 hours for dispersed cell adhesion and biofilms formation, respectively, at 30 ºC and 120 rpm (CERTOMAT® BS-1, Sartorius AG, Germany).

### 3.9. Sessile bacteria in an agitated reactor

These tests were performed using a glass chemostat biofilm reactor (2 L), at room temperature, aerated and magnetically agitated, similar to the one described by Ferreira et al. (2012). The reactor was inoculated with 500 mL of bacteria from an overnight culture with a specific medium (5.5 $g.L^{-1}$ glucose, 2.5 $g.L^{-1}$ peptone, 1.25 $g.L^{-1}$ yeast extract, 1.88 $g.L^{-1}$ monopotassium phosphate and 2.6 $g.L^{-1}$ sodium phosphate dibasic) with an initial number of cells of $4 \times 10^7$ $CFU.mL^{-1}$. This volume (500 mL) was added to 1.5 L of 8.5 $g.L^{-1}$ NaCl. The feeding process began 2 hours after inoculation. To allow biofilm formation instead of planktonic growth, the reactor was continuously fed with 0.2 $L.h^{-1}$ of a sterile solution with 55 $mg.L^{-1}$ glucose, 25 $mg.L^{-1}$ peptone, 12.5 $mg.L^{-1}$ yeast extract, 1.88 $g.L^{-1}$ monopotassium phosphate and 2.6 $g.L^{-1}$ sodium phosphate dibasic. The SS slides (2.0 cm × 2.0 cm × 0.1 cm) were placed vertically in contact with the bacterial suspension for 5 days. This set-up was used for experiments with *E. coli* CECT 434 and a food isolate (*P. oryzihabitans*). A schematic representation for the set-up when both strains were tested can be observed in Figure 3.3.

### 3.10. Scanning electron microscopy (SEM)

SS coupons of 24 h biofilms of *E. coli* CECT 434 and *P. oryzihabitans* were observed by SEM. Samples were washed with 8.5 $g.L^{-1}$ NaCl, then fixed with 3% $w.w^{-1}$ glutaraldehyde (Merck, Germany) in sodium cacodylate buffer 0.14 $mol.L^{-1}$ pH 7.2 for 10 min. (Gomes and Mergulhão, 2017). After this step, the samples were washed again with 8.5 $g.L^{-1}$ NaCl. Then the samples were dehydrated with ethanol (Fisher Scientific, UK) in a series of 50, 60, 70, 80, 90, and 2 × 100% ($v.v^{-1}$) for 10 min each. The dehydration was continued with hexamethyldisilazane (HMDS, Merck, Germany) at 50, 60, 70, 80, 90, and 2 × 100% ($v.v^{-1}$) prepared in ethanol 100%, for 10 min each (Gomes et al., 2013). All the coupons were then dried for 1 day. Samples were coated with a Au/Pd thin film, by sputtering, using the SPI Module Sputter Coater equipment. The SEM/EDS exam was performed using a High resolution (Schottky) Environmental Scanning Electron Microscope with X-Ray Microanalysis and Electron Backscattered Diffraction analysis: Quanta 400 FEG ESEM / EDAX Genesis X4M in high-vacuum mode at 15 kV.

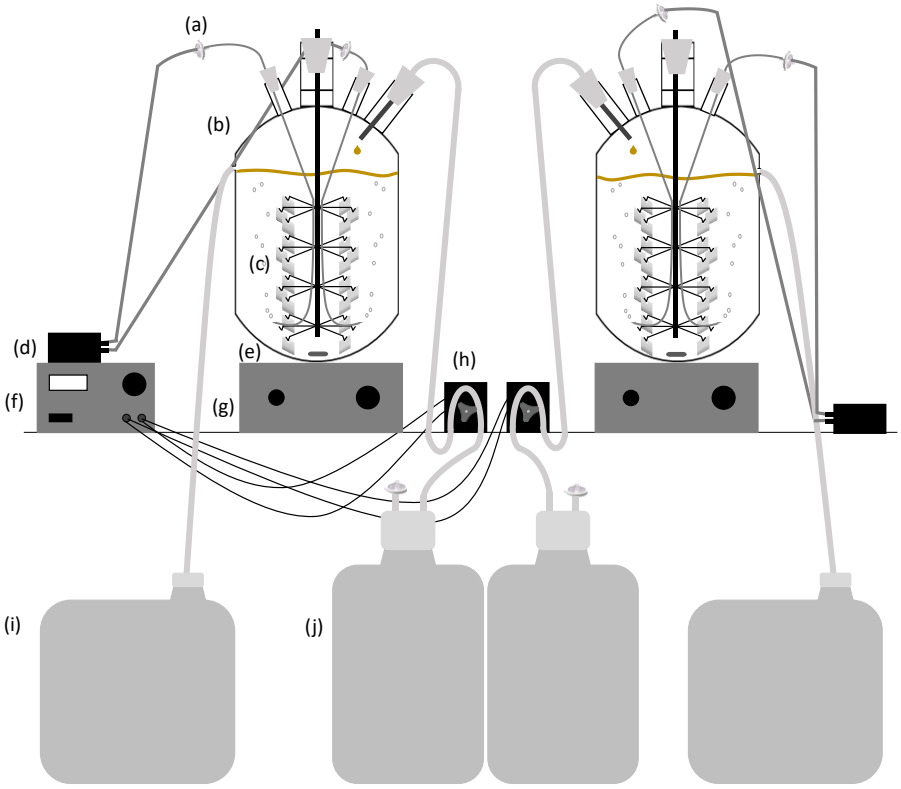


**Figure 3.3.** Schematic representation of the experimental set-up of two agitated reactors used to study *E. coli* CECT 434 and *P. oryzihabitans* biofilms. (a) air filter; (b) agitated reactor; (c) SS coupons; (d) air pump; (e) magnetic bar; (f) power supply; (g) magnetic stirrer; (h) peristaltic pump; (i) waste container; (j) feed container.

## 3.11. Optical coherence tomography (OCT)

The SS coupons obtained from the sessile tests in the agitated reactor after 5 days of *E. coli* CECT 434 and a food isolate (*P. oryzihabitans*) growth were observed by OCT. The 3D images were obtained using an OCT Ganymed from Thorlabs (Germany) with a center wavelength of 930 nm using ThorImage 4.2 software. The refractive index used was that of water (1.33) (Li et al., 2001).

## 3.12. Efficacy of disinfectants

### 3.12.1. Disinfectants studied

SH 13% ($w.w^{-1}$) was obtained from Acros Organics (Belgium) and vanillin (VN) was acquired from Sigma-Aldrich (Switzerland). Peracetic acid (PA) 38-40% ($w.v^{-1}$), HP 30% ($w.v^{-1}$), sodium bicarbonate (SB) and copper sulfate (CS) pentahydrate were obtained from Merck (Germany). Chlorine dioxide (CD) 2 $g.L^{-1}$ was provided by Loehrke (Germany). NEOW was produced in an electrolysis chamber (ECAse) developed by

Loehrke (Germany). A sterilized NaCl (2.5 $g.L^{-1}$) solution was used to produce NEOW with 300 ppm of free chlorine content. The ECAse consumed 26 $L.h^{-1}$ of brine and the overall flow was divided into 13 $L.h^{-1}$ of anolyte and 13 $L.h^{-1}$ of catholyte. Sodium dichloroisocyanurate (NaDCC) was acquired from Acros Organics (Belgium).

### 3.12.2. Minimum inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) determination

Bacteria were obtained from overnight cultures grown in 100 mL flasks with 25 mL of MHB (Merck, Germany), incubated at 30 ºC and 120 rpm (CERTOMAT® BS-1, Sartorius AG, Germany). The MIC was determined in 96-well flat-bottomed PS tissue culture plates with a lid (Orange Scientific, USA) using a total volume of 200 µL. In each microtiter plate well, 180 µL of bacterial inoculum ($O.D._{600nm}$ of 0.1) was added to 20 µL of increasing concentrations of disinfectants: SH, VN, PA, HP, CS, SB and the plates were incubated for 24 h at 30 °C and 120 rpm (CERTOMAT® BS-1, Sartorius AG, Germany). *E. coli* CECT 434 was used in the tests for all these disinfectants. A food isolate (*P. oryzihabitans*) was only tested for SH and PA. After 24 hours exposure to the disinfectants, the $OD_{600}$ was measured in a Synergy™ HT 96-well microplate reader (Biotek Instruments, Inc., USA) and the MIC was determined as the lowest concentration of the antimicrobial that inhibits the growth of the microorganism (Andrews, 2001, Yilmaz, 2012). To determine the MBC, the motionless drop method (Reed and Reed, 1948) was used on PCA plates (Merck, Germany). The plates were incubated overnight at 30 ºC after a neutralization step (adding sodium thiosulfate 0.1%) (Johnston et al., 2002). MBC was determined as the lowest concentration of an antimicrobial that kills a microorganism (Andrews, 2001, Yilmaz, 2012) after a 24 hours incubation period. At least three independent experiments were performed for each condition tested.

### 3.12.3. Combination of sodium hypochlorite with other disinfectants

These combinations were only studied for *E. coli* CECT 434. Disinfectant combinations were assayed using the previously described method (section 3.12.2) by applying 50% ($v.v^{-1}$) of each disinfectant in a total volume of 20 µL. SH concentration used in combination with the other disinfectants (VN, PA, HP, CS, SB) was half of the individual MIC (225 ppm). In the case of SH combined with HP and CS the disinfectants were applied at the following levels 50% ($v.v^{-1}$), 40% ($v.v^{-1}$) and 10% ($v.v^{-1}$), respectively (Cerioni et al., 2013). Additionally, the concentration used for CS in this combination

was constant: 1500 ppm (the concentration generally used for plant fumigation (Cerioni et al., 2009). At least three independent experiments were performed for each condition tested.

The presence or absence of synergism was determined by the calculation of the MIC ratio (Table 3.2). Where $MIC_c$ is the MIC of the compound in the combination and $MIC_i$ is the MIC of the compound used individually. If the MIC ratio is lower than 0.5 potentiation is occurring; if it is lower than 1 but higher than 0.5 it is considered a modest enhancement in antimicrobial activity; and if it is equal or higher than 1 it means that the combination is antagonistic.

### 3.12.4. Colony forming units (CFU) quantification

Bacteria were obtained from overnight cultures incubated at 30 ºC and 120 rpm (CERTOMAT® BS-1, Sartorius AG, Germany) and grown in 50 mL tubes with 20 mL of medium. For comparison of the artificial lettuce medium and MHB, both media were used.

An $O.D._{600nm}$ of 0.04 was obtained and the cells were placed in 96-well flat-bottomed PS tissue culture plates with a lid (Orange Scientific, Belgium) using a total volume of 200 µL (Borges et al., 2012). After an incubation period (30 ºC and 120 rpm (CERTOMAT® BS-1, Sartorius AG, Germany)) of 24 h the media with cells were removed and the wells were washed with 8.5 $g.L^{-1}$ NaCl that was discarded. 8.5 $g.L^{-1}$ NaCl (180 µL) was mixed with the disinfectant agent (20 µL). After 20 min of contact time (to have a medium exposure time to have a bactericidal effect (Adams et al., 1989)) the disinfectant was removed, and the wells were washed with 8.5 $g.L^{-1}$ NaCl. Finally, 8.5 $g.L^{-1}$ NaCl (250

**Table 3.2.** Calculation and significance of the MIC ratio (adapted from Rodin et al. (2005))

| MIC ratio | Value | Result |
|---|---|---|
| $\frac{MIC_c}{MIC_i}$ | $0 < \frac{MIC_c}{MIC_i} < 0.5$ | Potentiation |
| | $0.5 \leq \frac{MIC_c}{MIC_i} < 1$ | Modest enhancement |
| | $\frac{MIC_c}{MIC_i} \geq 1$ | Antagonism |

$MIC_c$ is the MIC of the compound in the combination and $MIC_i$ is the MIC of the compound when used individually

µL) was added, the wells were scraped 4 times and the volume was collected. To determine the CFU the motion drop method was used on PCA plates and the necessary dilutions were performed. Plates were incubated overnight, at 30 ºC. At least three independent experiments were performed for each condition tested.

### 3.12.5. Disinfection of dispersedly adhered cells and biofilms on coupons

After the incubation period for bacterial adhesion or biofilm formation (2 h or 24 h) described in section 3.8, the medium was removed and replaced by the disinfectant solution for 20 min (to have a medium exposure time to have a bactericidal effect (Adams et al., 1989)), at 30 ºC and 120 rpm (CERTOMAT® BS-1, Sartorius AG, Germany). Afterwards, the coupons were placed in 5 mL 8.5 $g.L^{-1}$ NaCl, the cells were removed by vigorously vortex (1 min) and the neutralization step was performed by dilution to sub-inhibitory concentrations (Johnston et al., 2002). The necessary dilutions were prepared to determine the CFU using the motion drop method on PCA plates (Reed and Reed, 1948). For SH, VN, PA, HP, CS and SB the concentrations chosen were MIC, 5× MIC. In the combination tests, the disinfectants concentration used were the ones defined by the individual tests in MIC determination (section 3.12.2). The food isolates were only tested for biofilm disinfection with SH and PA at 50 ppm. At least three independent experiments were performed for each condition tested.

The presence or absence of synergism was determined by the calculation of the log CFU reduction index (LR) (Table 3.3). Where LRc is the log CFU reduction of the compound in the combination and LRi is the log CFU reduction of the compound used individually. If the LRc is higher than the LRi it represents an enhancement of the disinfectant activity; if it is equal to LRi, a neutral effect is considered; and if it is lower than the LRi antagonism is present.

**Table 3.3.** Calculation and significance of the LR value

| **LR value** | **Result** |
|---|---|
| $LR_c > LR_i$ | Enhancement |
| $LR_c = LR_i$ | Neutral |
| $LR_c < LR_i$ | Antagonism |

$LR_c$ is the log CFU reduction of the compound in the combination and $LR_i$ is the log CFU reduction of the compound when used individually

### 3.12.6. Biofilm removal

These tests were only performed for the isolates. After the disinfection process described in the section 3.12.5, biofilm removal was assessed by epifluorescence microscopy using 4',6-diamidino-2-phenylindole (DAPI, Sigma, Portugal) for staining the total number of cells. Each slide was stained with 20 µL of DAPI at a concentration of 0.5 µg.mL$^{-1}$. After 10 min incubation in the dark the slides were mounted with non-fluorescent immersion oil on glass microscope slides. The slides were examined using an epifluorescence microscope (LEICA DMLB2) with a filter with the following characteristics: excitation filter 340-380 nm, dichromatic mirror of 400 nm and suppression filter LP 425. A minimum of 20 image series were acquired for each coupon and at least three independent experiments were performed for each condition tested.

### 3.12.7. Biofilm regrowth

To assess biofilm regrowth, after disinfectant exposure (section 3.12.5) coupons were placed in 48-wells flat-bottomed PS tissue culture plates (Thermo Fisher Scientific, Korea) with fresh MHB media. The plates were incubated for 24 hours, at 30 ºC and 120 rpm (CERTOMAT® BS-1, Sartorius AG, Germany). After this period the numbers of CFU were assessed by the motion drop method (Reed and Reed, 1948) on PCA plates. These tests were only performed for the isolates. At least three independent experiments were performed for each condition tested.

### 3.12.8. Time kill curves

The time kill curves were obtained with *E. coli* CECT 434 planktonic cells from overnight growth in MHB (30 °C and 120 rpm). The cells were centrifuged (4000 g, 15 min) and washed one time with 8.5 g.L$^{-1}$ NaCl. Afterwards, bacteria were resuspended in 8.5 g.L$^{-1}$ NaCl to obtain an O.D.$_{600}$ of 0.2. These cells were centrifuged again (4000 g, 15 min) and 8.5 g.L$^{-1}$ NaCl was replaced by the biocides (SH, NEOW, CD and NaDCC prepared in 8.5 g.L$^{-1}$ NaCl) at different concentrations (20, 50, 80 and 100 ppm of free chlorine). The range of concentrations chosen were based in the values usually applied in the MPV industry: 70-90 ppm (Rico et al., 2007, Goodburn and Wallace, 2013). 8.5 g.L$^{-1}$ NaCl was used as control. These solutions were placed in 96-well flat-bottomed PS tissue culture plates with a lid (Orange Scientific, USA) using a total volume of 200 µL. During two hours with interval times of 5 minutes, at 25 ºC and with agitation (Synergy™ HT, Biotek Instruments, Inc., USA) samples were collected and the necessary dilutions were

performed to determine CFU using the motion drop method on PCA (Merck, Germany) plates (Reed and Reed, 1948). The Gompertz model (Gompertz, 1825) was used to determine the time kill kinetic parameters related to the over time CFU variation. This kinetic model has already been applied to characterize a biocide kinetic action by Garthright (1991). Equation 3.2 was applied to determine the theoretical model:

$$CFU(t_m) = CFU_0 + (CFU_f - CFU_0) \times e^{-e^{[k_{CFU} \times (\lambda_{CFU} - t_m) + 1]}} \quad (3.2)$$

Where $CFU(t_m)$, $CFU_0$ and $CFU_f$ correspond to: the CFU at a defined time $t_m$ in min, the initial value of the CFU (the upper asymptote curve), and the CFU final value, respectively; $k_{CFU}$ is the kinetic constant (log CFU.mL$^{-1}$.min$^{-1}$) and represents the maximum antimicrobial rate, $\lambda_{CFU}$ is the lag time for antimicrobial action (min), $t_m$ is the time (min). The kinetic parameters ($k_{CFU}$ and $\lambda_{CFU}$) were determined using the Solver supplement of Microsoft Excel 2016. The quality of the model was evaluated through the coefficient of determination ($R^2$) and with RMSE (root mean square error) determination.

### 3.12.9. Tests with sessile bacteria in an agitated reactor

After biofilm formation (section 3.9), the SS slides were transferred to closed sterile flasks. For *E. coli* CECT 434 the following conditions were tested: control with 8.5 g.L$^{-1}$ NaCl, SH, NEOW, CD, NaDCC and PA at 50 ppm. For *P. oryzihabitans* the conditions tested were: control with 8.5 g.L$^{-1}$ NaCl, SH and PA at 50 ppm. The concentration chosen (50 ppm) was based on the requisites of the MPV industry to reduce the concentrations usually applied (Rico et al., 2007, Goodburn and Wallace, 2013). The disinfectants solutions were prepared in 8.5 g.L$^{-1}$ NaCl.

The flasks were placed in magnetic shakers at 25 ºC, for 20 min (to have a medium exposure time to have a bactericidal effect (Adams et al., 1989)). Afterwards, the slides were subjected to a neutralization step by diluting the biocides to sub-inhibitory levels (Johnston et al., 2002). For that, the SS slides were inserted in 50 mL sterile tubes with 10 mL of 8.5 g.L$^{-1}$ NaCl. The tubes were subjected to vigorous (100% of maximum power) vortex (VWR, Portugal) during 2 min, and the necessary dilutions were performed to determine the variation in the number of CFU using the motion drop method on PCA (Merck, Germany) (Reed and Reed, 1948). A schematic representation of the disinfection and further steps is represented in Figure 3.4.

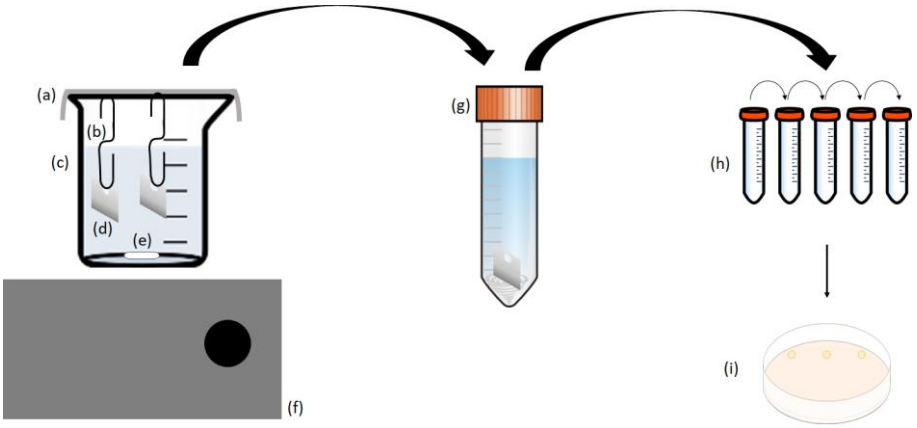


**Figure 3.4.** Schematic representation of the procedure used for SS slides disinfection and further steps to assess disinfection. (a) aluminum foil; (b) metal clips; (c) glass flask; (d) SS coupon; (e) magnetic bar; (f) magnetic stirrer; (g) 50 mL sterile tube with 8.5 $g.L^{-1}$ NaCl; (h) 15 mL sterile tubes with 8.5 $g.L^{-1}$ NaCl; (i) PCA.

### 3.12.10. Reactive oxygen species

Dichloro-dihydro-fluorescein diacetate (DCFH-DA) assay was used for the determination of reactive oxygen species (ROS). In this method, DCFH-DA is oxidized by ROS resulting in a fluorescent molecule: 2'7'-dichlorofluorescein diacetate (Jambunathan, 2010). The solution was acquired at a concentration ≥ 97% (Sigma Aldrich, USA) and was prepared in absolute ethanol (Fisher Scientific, UK) (Rastogi et al., 2010). *E. coli* CECT 434 from overnight growth in MHB (30 °C and 120 rpm) were centrifuged (4000 g, 15 min) and washed one time with 8.5 $g.L^{-1}$ NaCl. Afterwards, bacteria were resuspended in 8.5 $g.L^{-1}$ NaCl to obtain an $O.D._{600}$ of 0.2. These cells were centrifuged again (4000 g, 15 min) and 8.5 $g.L^{-1}$ NaCl was replaced by biocides (SH, NEOW, CD and NaDCC prepared in 8.5 $g.L^{-1}$ NaCl) in the concentrations tested (20, 50, 80 and 100 ppm of free chlorine). *E. coli* control (only cells) and biocides control (without cells) were also prepared in 8.5 $g.L^{-1}$ NaCl. Before adding the previous prepared and described solutions to a 96-well flat-bottomed PS microplate (Orange Scientific, USA), a solution of DCFH-DA was added to the same 96-well PS microplate in a volume of 20 µL (5 µM as final concentration in 200 µL final volume) (Rastogi et al., 2010). Then, control (only cells), biocide solutions (20, 50, 80 and 100 ppm) and biocides (20, 50, 80 and 100 ppm) with cells were placed in the same microplates (180 µL). The fluorescence was measured (optics position on top) during 30 min with interval times of 2 min at 25 ºC using a Synergy™ HT fluorescence reader (Biotek Instruments, Inc., USA). Wavelength of

485/20 and 528/20 were used for excitation and emission, respectively (Rosenkranz et al., 1992).

### 3.12.11. Chemical stability of chlorine-based solutions

The stability of the biocidal solutions, *i.e.* over time depletion of free chlorine, was evaluated with a free chlorine portable meter HI 93701 (Hanna Instruments, England) at 5, 25 and 30 ºC, during 200 days. SH, NEOW, CD and NaDCC were prepared at 100 ppm in a total volume of 100 mL for each temperature.

The Gompertz model (Gompertz, 1825) was used to determine the kinetic parameters related to the overtime variation of free chlorine levels. Equation 3.3 was applied to determine the theoretical model:

$$FC(t_d) = FC_0 + (FC_f - FC_0) \times e^{-e^{[k_{FC} \times (\lambda_{FC} - t_d) + 1]}} \quad (3.3)$$

Where $FC(t_d)$, $FC_0$ and $FC_f$ correspond to: the free chlorine (FC) at a determined time, $t_d$, the initial value of the FC (the upper asymptote curve), and the FC final value, respectively; $k_{FC}$ is the kinetic constant (ppm.days$^{-1}$) that represents the maximum chlorine loss rate, $\lambda_{FC}$ is the lag time for chlorine loss (days), $t_d$ is the time (days). The kinetic parameters ($k_{FC}$ and $\lambda_{FC}$) were determined using the Solver supplement of Microsoft Excel 2016. The quality of the model was evaluated through the coefficient of determination ($R^2$) and with RMSE determination.

## 3.13. Development of an artificial lettuce medium

To stablish the artificial medium the composition was based on the chemical composition described for lettuces (Oke et al., 2012). The reagents used and the percentages applied are present in Table 3.4.

*E. coli* CECT 434 was selected for this study and was obtained from overnight cultures grown in 50 mL tubes with 20 mL of medium. MHB (Merck, Germany) was used for comparison as it is the medium proposed by CLSI (2005) to test antimicrobial activity. The tubes were incubated at 30 ºC and 120 rpm (CERTOMAT® BS-1, Sartorius AG, Germany). *E. coli* $O.D._{600nm}$ was adjusted to 0.1 in 1 L Erlenmeyer's with 250 mL of each medium. Both Erlenmeyer's were incubated at 30 ºC and 120 rpm (CERTOMAT® BS-1,

**Table 3.4.** Lettuce broth (LTB) composition and concentration of each component

| Component | Ingredient | Concentration % (w.v$^{-1}$) |
|---|---|---|
| Water | Distilled water | 95 |
| Carbohydrates | Glucose (Chem-Lab, Belgium) | 2.8 |
| Proteins | Soy peptone (Liofilchem, Italy) | 1.3 |
| Ashes | Sodium bicarbonate ( Merck, Germany) | 0.9 |

Sartorius AG, Germany). Over time, samples were taken for measuring the O.D.$_{600nm}$ and for CFU determination. To determine the CFU the motion drop method on PCA was used and plates were incubated overnight at 30 ºC. The necessary dilutions were performed. At least three independent experiments were performed for each condition tested. To determine the growth rate (μ, h$^{-1}$) equation 3.4 was used while the generation or doubling time ($T_d$, h) was assessed according to equation 3.5 (Maier, 2009).

$$\mu = \frac{(\log \mathrm{CFU.mL^{-1}})_i - (\log \mathrm{CFU.mL^{-1}})_0}{T_i - T_0} \quad (3.4)$$

$$T_d = \frac{\ln 2}{\mu} \quad (3.5)$$

Where *i* represents the end of the exponential phase, *0* represents the beginning of the exponential phase and *T* is time (h).

Samples of *E. coli* CECT 434 in LTB and MHB were characterized by SEM. For that, *E. coli* CECT 434 cells had to be adhered in a surface. Therefore, cells from overnight growth were adjusted to an O.D.$_{600nm}$ of 0.1 in 100 mL flasks with 25 mL of each medium and incubated at 30 °C and 120 rpm (CERTOMAT® BS-1, Sartorius AG, Germany). After 5 h 3 mL of each sample were placed on SS surfaces during 2 h. After this adhesion step, samples were prepared according to section 3.10.

To compare both media, the MIC and MBC were determined (as described in section 3.12.2) for SH and PA using LTB and MHB as media. Additionally, biofilm formation was evaluated in 96-well flat-bottomed PS tissue culture plates with CV staining for biomass determination (section 3.7.1) and CFU assessment to determine the culturable cells (described in section 3.7.2 and 3.12.4).

## 3.14. Statistical analysis

Statistical analysis was performed using GraphPad Prism, version 5.0 for Windows. Statistical significance values were evaluated using a two-way ANOVA (Bonferroni post-tests to compare replicate means by row). Statistical calculations were based on a confidence level of ≥ 95% ($P < 0.05$ was considered statistically significant).

# 4

## RESULTS AND DISCUSSION

In this section all the results obtained, according to the aims of the work, will be presented and discussed.

## 4.1. Efficacy of disinfectants used individually and in combination

Antimicrobial products, particularly chlorine, have been used for disinfection in food industry (Davidson and Harrison, 2002, Gerba, 2015). Although disinfection with chlorine is widespread, there is a global concern on developing alternative disinfection strategies and on minimizing its environmental and public health impacts (WHO, 2011). In fact, chlorine can react with organic compounds to produce disinfection by-products that have potential carcinogenic effects (Simões and Simões, 2013). Therefore, optimization on its use for disinfection is required. No chlorine is probably an unreachable target, based on the work of Kim et al. (2009) and considering that this agent is efficient in the control of anaerobic bacteria and biofilms. Therefore, the present study aimed to develop strategies to reduce the use of chlorine in the control of *E. coli* CECT 434 planktonic and sessile growth.

### 4.1.1. MIC and MBC determination

SH and other selected disinfectants were tested and the individual MIC and MBC were determined (Table 4.1). The inhibitory and bactericidal effect for which a lower concentration of the disinfectants was necessary was, in an ascending order of concentrations: CD < SH < PA < HP < CS.

**Table 4.1.** MIC and MBC obtained for the individual disinfectants

| Disinfectant | MIC (ppm) | MBC (ppm) |
|---|---|---|
| Sodium hypochlorite | 450 ± 74 [a] | 450 ± 74 [a] |
| Chlorine dioxide | 200 ± 0 [b] | 200 ± 0 [b] |
| Peracetic acid | 460 ± 0 [a] | 530 ± 91 [c] |
| Hydrogen peroxide | 510 ± 41 [c] | 540 ± 0 [d] |
| Copper sulfate pentahydrate | 6740 ± 574 [d] | 6990 ± 0 [e] |
| Vanillin | >685 [e] | >685 [f] |
| Sodium bicarbonate | >7560 [f] | >7560 [g] |

Values are presented as the mean (ppm) ± standard deviation for at least three independent experiments. Different letters within the same column represent statistically different values ($P < 0.05$).

The MIC and MBC of SH was 450 ppm. When comparing with previous studies, the MIC of SH was higher than the value obtained by Cerioni et al. (2009) (300 ppm) although in the same order of magnitude. This is arguably due to the different microorganisms tested (Russell, 2003). In fact, Cerioni et al. (2009) used a filamentous fungi (*Penicillium digitatum*). Also, the methods used to determine the MIC were different (Halliwell, 2006, Cerioni et al., 2009). Abadias et al. (2011) used 100 ppm SH and only achieved 1 log $CFU.mL^{-1}$ reduction of *E. coli*. Penna et al. (2001) obtained inhibitory concentrations in the range of 150-1120 ppm for *E. coli*. Heling et al. (2001) needed 1800 ppm and 28500 ppm to inhibit and have a bactericidal effect against *Enterococcus faecalis*, respectively. In a more recent work, Cerioni et al. (2013) determined MIC of SH for *Penicillium expansum* of 3700 ppm. For CD, the minimum concentration necessary to inhibit *E. coli* was 200 ppm. This disinfectant was already used by Maillard (2011) to test its sporicidal efficacy against *Bacillus cereus*. The author obtained a MBC of 200 ppm for spores of *B. cereus*, which is similar to the MBC and MIC value obtained for *E. coli* in the present study. The main drawback in the industrial use of this biocide is the maximum FDA (FDA, 2013) allowed concentration (3 ppm). This is a very low value and not suitable to promote significant reduction in the microbiological load of vegetables (Ölmez and Kretzschmar, 2009). Moreover, this biocide can also induce alterations in the organoleptic properties of fresh food products as it was demonstrated by Mahmoud and Linton (2008) on lettuce leaves when CD was applied in the gas form at 5 ppm. PA is another oxidizing agent that had a MIC of 460 ppm, similar to the MIC of SH. Bridier et al. (2011) determined a MBC of PA of 7 ppm for *E. coli* PHL 628 which is much lower than the value obtained in the present study (530 ppm). On the other hand Penna et al. (2001) obtained a MIC of 2280 ppm which is 5 times higher than the value obtained in this study. These results clearly reinforce that antimicrobial susceptibility is dependent on the microbial species/strains and on the methods used (Bridier et al., 2011). The mode of action of PA is not known but it is suggested that this acid disrupts the chemiosmotic function of the lipoprotein cytoplasmic membrane or causes rupture of cell walls (Kitis, 2004).

For HP the MIC value obtained was 510 ppm. Cerioni et al. (2013) determined the MIC of HP for *Penicillium expansum* obtaining a high value of 13600 ppm, as well as Cerioni et al. (2009) that obtained 10200 ppm. Miyasaki et al. (1986) described that the MBC of HP varies from 25 to 340 mM for *Haemophilus aphrophilus, Eikenella corrodens* and

*Capnocytophaga gingivalis*, respectively. Pericone et al. (2000) could not determine the MIC of HP, but obtained a MBC of 510 ppm against *E. coli* RS218 which is close to the value obtained in the present study (540 ppm). The bactericidal or bacteriostatic effect of HP on the microorganisms can be justified by the fact that this disinfectant is an oxidizer and can form toxic species that are responsible for its antimicrobial properties (Ölmez and Kretzschmar, 2009). In the case of CS, several authors (Cerioni et al., 2009, Finnegan et al., 2010, Cerioni et al., 2013) did not determined the MIC but stipulated that it was 1500 ppm, the concentration generally used for plant fumigation (Halliwell, 2006, Cerioni et al., 2009). In this study a MIC of 6740 ppm was obtained.

The MIC and MBC of VN were not determined, since the maximum concentration that could be tested (685 ppm, due to solubility limitations) was not sufficient to inhibit or kill *E. coli*. VN has low water solubility and this is a significant disadvantage for its application (Karathanos et al., 2007). However, there are studies demonstrating the antimicrobial activity of VN. Fitzgerald et al. (2004) found that the MIC of VN was 2280 ppm for *E. coli*, when this phytochemical was prepared in ethanol. Abadias et al. (2011) prepared the solution in acetic acid and achieved 1 log $CFU.mL^{-1}$ reduction of *E. coli* with VN at 12 000 ppm. Likewise, the MIC and MBC were not determined for SB. In fact, the solubility of SB in water is also a problem for food related disinfection. Miyasaki et al. (1986) already demonstrated that a very high concentration of SB was necessary to achieve an inhibitory (1930-15300 ppm, depending on the microorganism) or bactericidal effect (15300-61200 ppm). Additionally, Abadias et al. (2011) concluded that 99970 ppm SB caused no *E. coli* reduction.

Overall, PA and CD were the most promising alternative disinfectants to SH ($P < 0.05$) to inhibit and to eliminate *E. coli* in suspension. When comparing the MIC and the MBC values, only those for CS, HP and PA were different, although the differences were not statistically significant ($P > 0.05$). The significant action of CD was probably due to its low pH value (0.96). Also, PA had a pH of 4.00. The other disinfectants had pH values near neutrality (7.07, 7.00, 5.50, 7.00 and 6.50 for SH, HP, CS, SB and VN, respectively). These pH values do not reflect the mode of action of the disinfectants.

### 4.1.2. Combination of sodium hypochlorite with other disinfectants

After the individual MIC and MBC determinations, the disinfectants were combined with 225 ppm of SH in order to ascertain their putative antimicrobial potentiation (Table 4.2).

The inhibitory and bactericidal effect for which a lower concentration of disinfectants was necessary was, in an ascending order: CD < HP + CS < PA < CS < HP. The combination of SH with CD promoted modest enhancement on antimicrobial activity. Nevertheless, the difference between the MIC assayed individually or in combination with SH was not statistically significant ($P > 0.05$). The results obtained for PA when combined with SH seem very promising. Indeed, of all the combinations tested this was the one with the most promising results ($P < 0.05$), as the concentration of PA in the combination, necessary to have a bactericidal effect, was reduced 3-fold when compared to the individual tests. The combination of SH with HP and CS (1500 ppm) potentiated its antimicrobial activity. The concentration of HP was greatly reduced ($P < 0.05$), apparently due to the presence of copper. Cerioni et al. (2009) demonstrated that copper acts as a mediator of HP inducing damage in *E. coli*. This process is irreversible and affects the respiratory chain, with the consequent loss of bacterial viability (Cerioni et al., 2009). Furthermore, when HP is combined with metal ions like copper, the Fenton and Haber-Weiss reactions occur and HP is converted to the strongly reactive hydroxyl radical (Klaunig and Kamendulis, 2008). However, the application of copper has limitations since concentrations ranging from 0.6 to 2.4 ppm have been reported as 96 h LC50 median lethal concentration values for juvenile *Penaeus monodon* (Chen and Lin, 2001). In the present study the CS MIC used was greatly reduced ($P < 0.05$) in the combination with

**Table 4.2.** MIC and MBC obtained for different compounds when combined with SH 225 ppm

| Disinfectant | MIC (ppm) | MBC (ppm) | MIC ratio | Result |
|---|---|---|---|---|
| Chlorine dioxide | 100 ± 0 [a] | 100 ± 0 [a] | 0.50 | Modest enhancement |
| Peracetic acid | 150 ± 0 [b] | 200 ± 91 [b] | 0.33 | Potentiation |
| Hydrogen peroxide | 410 ± 0 [c] | 1410 ± 0 [c] | 0.80 | Modest enhancement |
| Copper sulfate pentahydrate | 2000 ± 500 [d] | 2500 ± 0 [d] | 0.30 | Potentiation |
| Hydrogen peroxide (with copper sulfate pentahydrate) | 140 ± 0 [e] | 230 ± 24 [e] | 0.27 | Potentiation |
| Vanillin | >335 [f] | >335 [f] | - | - |
| Sodium bicarbonate | >3780 [g] | >3780 [g] | - | - |

Values are presented as the mean (ppm) ± standard deviation for at least three independent experiments. Different letters within the same column represent statistically different values ($P < 0.05$).

SH. The results demonstrate that the CS + SH + HP combination can be advantageous in the control of planktonic *E. coli*. The enhancement of the inhibitory or bactericidal action of SH can be due to the fact that the disinfectants target the cell wall, causing structural changes or penetrate the cell and attack intracellular targets (Denyer, 1995). According to Denyer (1995), copper ions act in the cytoplasmatic membrane and HP and PA act in the cytoplasm, inhibiting catabolic and anabolic processes.

### 4.1.3. Disinfection of dispersedly adhered cells and biofilms

In the food industry, complete biofilm eradication is not always the objective but rather a logarithmic reduction (Cerf et al., 2010). Moreover, the microbial contaminants are not only in the planktonic phase, but also as cells dispersedly adhered on the surfaces and as biofilm structures (Simões et al., 2010b). Therefore, the selected disinfectants were tested for their ability to kill dispersedly adhered cells (Figure 4.1) and biofilms (Figure 4.2) from SS and PS surfaces. When comparing the materials tested, it is possible to observe that PS surface had a higher dispersedly cell adhesion ($4 \times 10^6$ CFU.cm$^{-2}$ in PS *vs* $1 \times 10^6$ CFU.cm$^{-2}$ in SS, $P > 0.05$) and biofilm development ($1 \times 10^7$ CFU.cm$^{-2}$ in PS *vs* to $3 \times 10^6$ CFU.cm$^{-2}$ in SS, $P < 0.05$).

According to Simões et al. (2007) adhesion is higher when both cell and substratum surfaces are hydrophobic. In this study, *E. coli* (Patel et al., 2011), SS 316 (Simões et al., 2007) and PS (Simões et al., 2010a, Machado et al., 2011) surfaces are hydrophobic. Therefore, adhesion was favored by the thermodynamic interactions established between cell and substratum surfaces. However, the hydrophobicity of SS (-55.1 mJ.m$^2$) (Simões et al., 2007) and PS (-55.2 mJ.m$^{-2}$) (Machado et al., 2011) was not, apparently, the main aspect causing the different cell densities.

The use of the selected chemicals demonstrated that their disinfecting potential was higher in adhered cells than in biofilms. In fact, *E. coli* already demonstrated increased antimicrobial resistance in biofilms (Ntsama-Essomba et al., 1997). Observing the results for dispersedly adhered cells (Figure 4.1), the removal from PS surfaces is less efficient than on SS. On SS surfaces (Figure 4.1a), all the disinfectants were effective except: VN at 685 and 3425 ppm, that promoted reductions of 1.2 ($P > 0.05$) and 2.2 log CFU.cm$^{-2}$ ($P < 0.05$), respectively; and SB 7560 ppm that promoted 0.26 log CFU.cm$^{-2}$ reduction

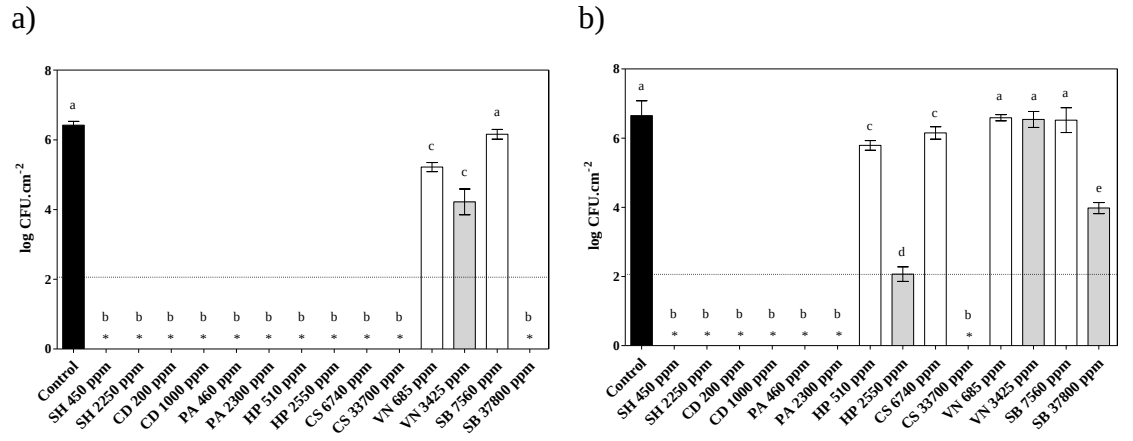


**Figure 4.1.** Log CFU.cm$^{-2}$ reduction achieved after the application of the individual disinfectants at their MIC and 5 × MIC against dispersedly adhered cells on SS (a) and on PS (b). The line indicates the method detection limit (2.06 log CFU.cm$^{-2}$). The symbol * represents that no CFU was detected. Different letters represent statistically different values ($P < 0.05$). The error bars represent the standard deviation. SH – sodium hypochlorite; HP – hydrogen peroxide; CS – copper sulfate; PA – peracetic acid; CD – chlorine dioxide; VN – vanillin; SB – sodium bicarbonate.

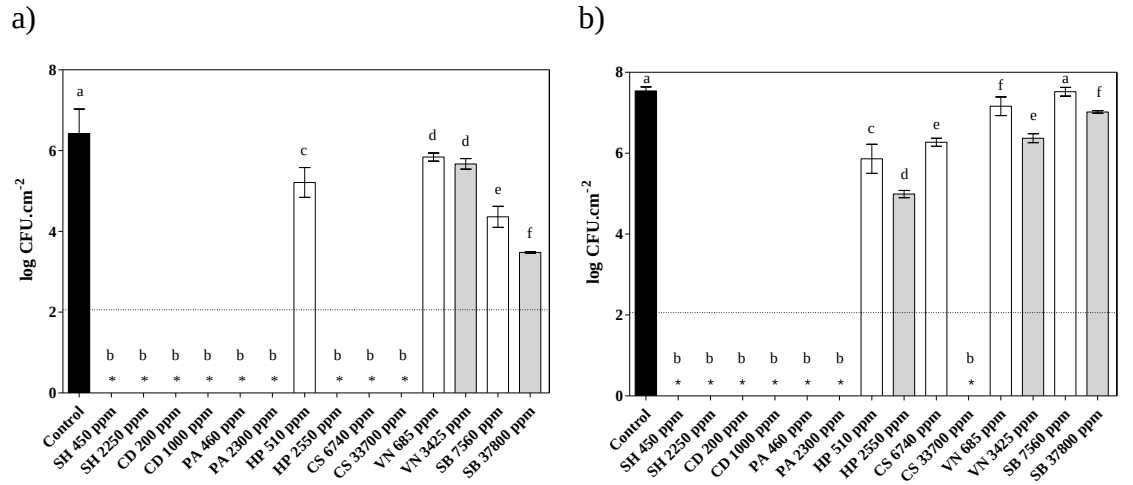


**Figure 4.2.** Log CFU.cm$^{-2}$ reduction achieved after the application of the individual disinfectants at their MIC and 5 × MIC against biofilms formed on SS (a) and on PS (b). The line indicates the method detection limit (2.06 log CFU.cm$^{-2}$). The symbol * represents that no CFU was detected. Different letters represent statistically different values ($P < 0.05$). The error bars represent the standard deviation. SH – sodium hypochlorite; HP – hydrogen peroxide: CS – copper sulfate; PA – peracetic acid; CD – chlorine dioxide; VN – vanillin; SB – sodium bicarbonate.

($P > 0.05$). On the PS surfaces (Figure 4.1b) SH, PA and CD completely killed the cells. As for HP, CS, VN and SB they were not significantly effective ($P > 0.05$). Only when the concentrations were increased from the MIC to 5 × MIC a significant reduction was

obtained: HP at 2550 ppm caused 4.58 log CFU.cm$^{-2}$ reduction ($P < 0.05$), CS completely eliminated *E. coli* ($P < 0.05$) and SB at 37800 ppm reduced 2.67 log CFU.cm$^{-2}$ ($P < 0.05$). VN at 3425 ppm caused no significantly different ($P > 0.05$) result from VN 685 ppm. Concerning biofilm control (Figure 4.2), HP, VN and SB were not significantly efficient ($P > 0.05$) in controlling biofilms on the SS surfaces (Figure 4.2a). When the concentration of HP was increased to 5 × MIC complete log CFU.cm$^{-2}$ reduction was achieved ($P < 0.05$). For the PS surface (Figure 4.2b), VN at 685 ppm and SB were also not efficient ($P > 0.05$) in biofilm control. Only by increasing the concentration of VN to 5 × MIC a 1.17 log CFU.cm$^{-2}$ reduction was achieved ($P < 0.05$). HP at 510 and 2550 ppm caused 1.68 and 2.55 log CFU.cm$^{-2}$ reduction, respectively ($P < 0.05$). As for CS at 6740 ppm, a 1.27 log CFU.cm$^{-2}$ reduction was achieved ($P < 0.05$), and 33700 ppm of CS were required to completely eradicate *E. coli* biofilm ($P < 0.05$). However, the application of copper has limitations for concentrations between 0.6 and 2.4 ppm (Chen and Lin, 2001). The findings on biofilm control with PA are similar to those of (Martín-Espada et al., 2014), when they achieved total eradication using 2660 ppm PA against *P. aeruginosa* biofilms formed on PS. Abadias et al. (2011) achieved 4 log CFU.mL$^{-1}$ *E. coli* reduction with 75 ppm PA on apples. In fact, the surface disinfection properties of PA were already proposed by Carpentier and Cerf (1993).

In general, SH, PA and CD were the best disinfectants while HP, SB and CS were less efficient in the killing of both dispersedly adhered cells and biofilms from both surfaces. The killing of dispersedly adhered cells with SB was only possible on SS surfaces. This is an interesting compound as it is a non-toxic food additive (Malik and Goyal, 2006).

The results obtained for the combination of disinfectants against dispersedly adhered cells and biofilms are shown in Figure 4.3 and 4.4, respectively. For both surfaces tested and type of tests performed (dispersedly adhered cells and biofilms) the results were similar, *i.e.* when the disinfectants were combined with SH at 225 ppm the elimination was efficient for all the tested conditions ($P < 0.05$) ($LR_c = LR_i$), except for HP and VN. These two combinations were antagonistic ($LR_c < LR_i$), as when SH at 225 ppm was applied alone it reduced 3 log CFU.cm$^{-2}$ ($P<0.05$) and when SH was combined with the other disinfectants (HP and VN) log CFU.cm$^{-2}$ reduction was lower. It is important to note that SB combined with SH was effective for complete control of dispersedly adhered cells and biofilms from both PS and SS surfaces ($LR_c > LR_i$). This is an unexpected result based

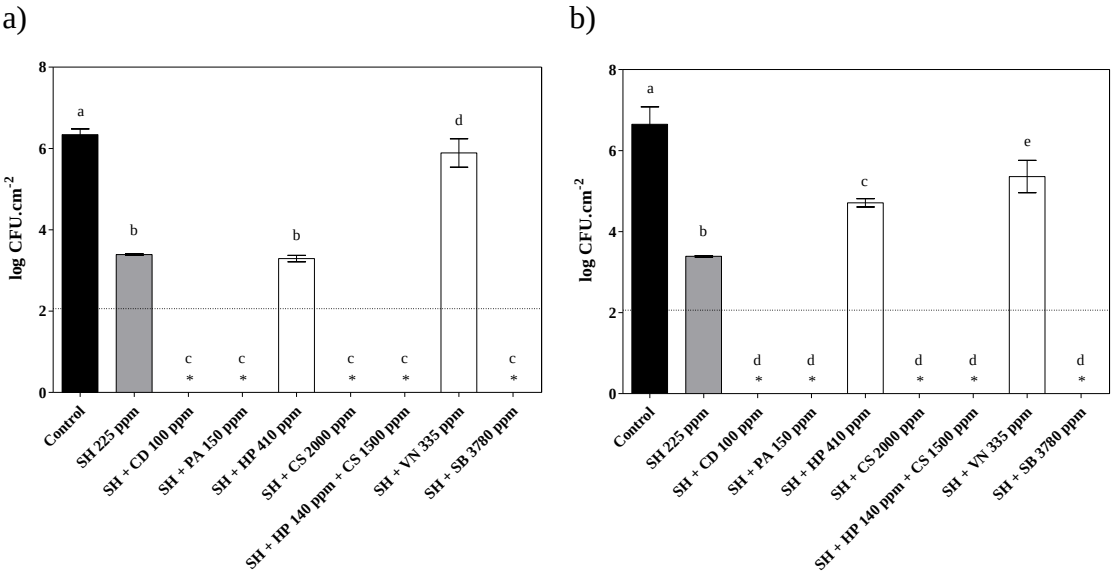


**Figure 4.3.** Log CFU.cm$^{-2}$ reduction achieved after the application of the combined disinfectants against dispersedly adhered cell on SS (a) and on PS (b). The line indicates the method detection limit (2.06 log CFU.cm$^{-2}$). The symbol * represents that no CFU was detected. Different letters represent statistically different values ($P < 0.05$). The error bars represent the standard deviation. SH – sodium hypochlorite; HP – hydrogen peroxide: CS – copper sulfate; PA – peracetic acid; CD – chlorine dioxide; VN – vanillin; SB – sodium bicarbonate.

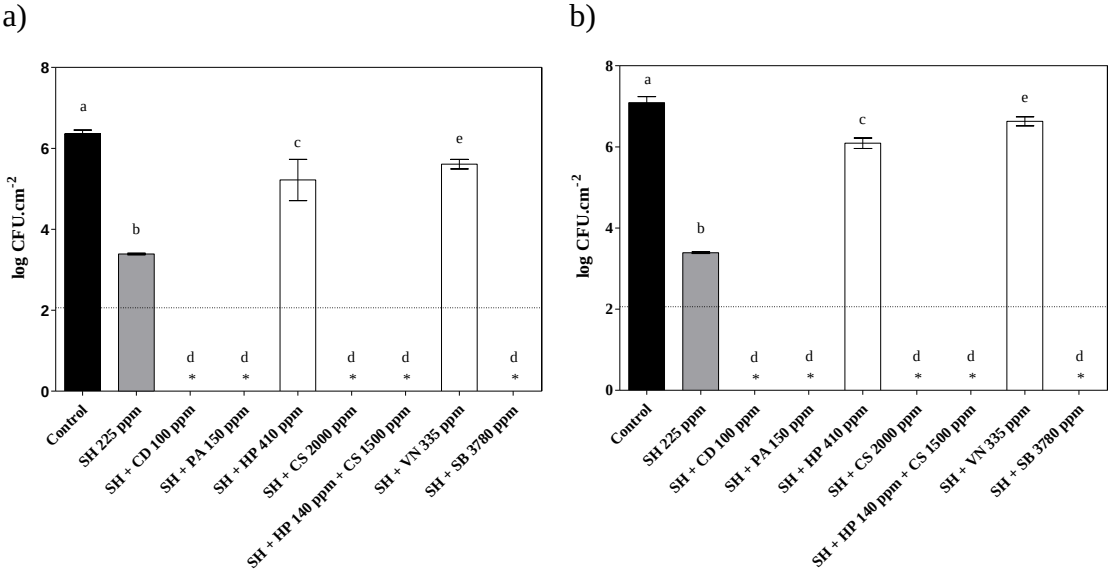


**Figure 4.4.** Log CFU.cm$^{-2}$ reduction achieved after the application of the combined disinfectants against biofilms formed on SS (a) and on PS (b). The line indicates the method detection limit (2.06 log CFU.cm$^{-2}$). The symbol * represents that no CFU was detected. Different letters represent statistically different values ($P < 0.05$). The error bars represent the standard deviation. SH – sodium hypochlorite; HP – hydrogen peroxide: CS – copper sulfate; PA – peracetic acid; CD – chlorine dioxide; VN – vanillin; SB – sodium bicarbonate.

on the performance of SB when used alone and when combined with SH against planktonic cells. In biofilms the microbial growth rate is reduced and there are less nutrients available, which can explain why SB was more efficient against the sessile cells (Gawande et al., 2008). In practice SH and SB demonstrated to be the most promising combination to be used in the disinfection of food surfaces. In fact, the concentration of SH can be reduced and SB has a GRAS status and is already applied in the disinfection of food surfaces (Palou et al., 2001, Malik and Goyal, 2006).

PA and CD, alone and combined with SH, were the most effective biocides to control planktonic and sessile *E. coli*. Interestingly, SB was potentiated by SH in the control of sessile *E. coli*. Taking into account that SH is considered a major risk for the formation of carcinogenic and mutagenic products (European Comission, 2007), the overall results demonstrate that the reduction of SH concentration in disinfection is possible using alternative biocide combinations.

## 4.2. Comparative efficacy of four chlorine-based disinfectants

In the fresh-cut vegetables industry, equipment and water disinfection is crucial to control microbial growth and prevent biofilm formation. SH is the biocide commonly applied for that purposes. With the aim to reduce the use of SH in disinfection processes, three chlorine-based biocides, NEOW, CD and NaDCC, were evaluated as alternatives in the control of *E. coli* CECT 434. For this evaluation the disinfectants efficacy was tested with time kill curves, biofilm elimination, reactive oxygen species formation. The biocides chemical stability was also assessed.

### 4.2.1. Time kill curves

Time kill curves of each biocide were determined in order to evaluate their antimicrobial effects (Figure 4.5). Kinetic parameters related to antimicrobial activity were obtained from the time kill curves (Table 4.3): $k_{CFU}$ that represents the maximum antimicrobial rate, $\lambda_{CFU}$ which is the time for antimicrobial action and the microbial elimination evaluated by the log CFU.mL$^{-1}$ decrease. From the model curves determined, the $R^2$ (close to 1) and RMSE values, the Gompertz model demonstrated to be suitable to describe the antimicrobial activity of the selected biocides. From the biocides tested, SH is the one requiring less time to have antimicrobial action (Figure 4.5). This is reinforced by the kinetic data (Table 4.3) where SH has the shortests $\lambda_{CFU}$. NEOW follows SH with

the shortest time, while NADCC is the biocide requiring higher time to have antimicrobial effects. Additionally, the action of CD and NaDCC was very similar ($P > 0.05$) as can be seen from Figure 4.5 and Table 4.3. Furthermore, by observing the $k_{CFU}$ values it is possible to conclude that among all the biocides NaDCC is the one with the highest antimicrobial rates. In a decreasing order of antimicrobial rates: NaDCC > CD > SH > NEOW. However, despite the fact that NEOW is the biocide with the lowest antimicrobial rates, 120 min after exposure to 80 and 100 ppm it caused the highest log CFU.mL$^{-1}$ reductions (0.779 and 1.052 at 80 and 100 ppm, respectively). Under these conditions

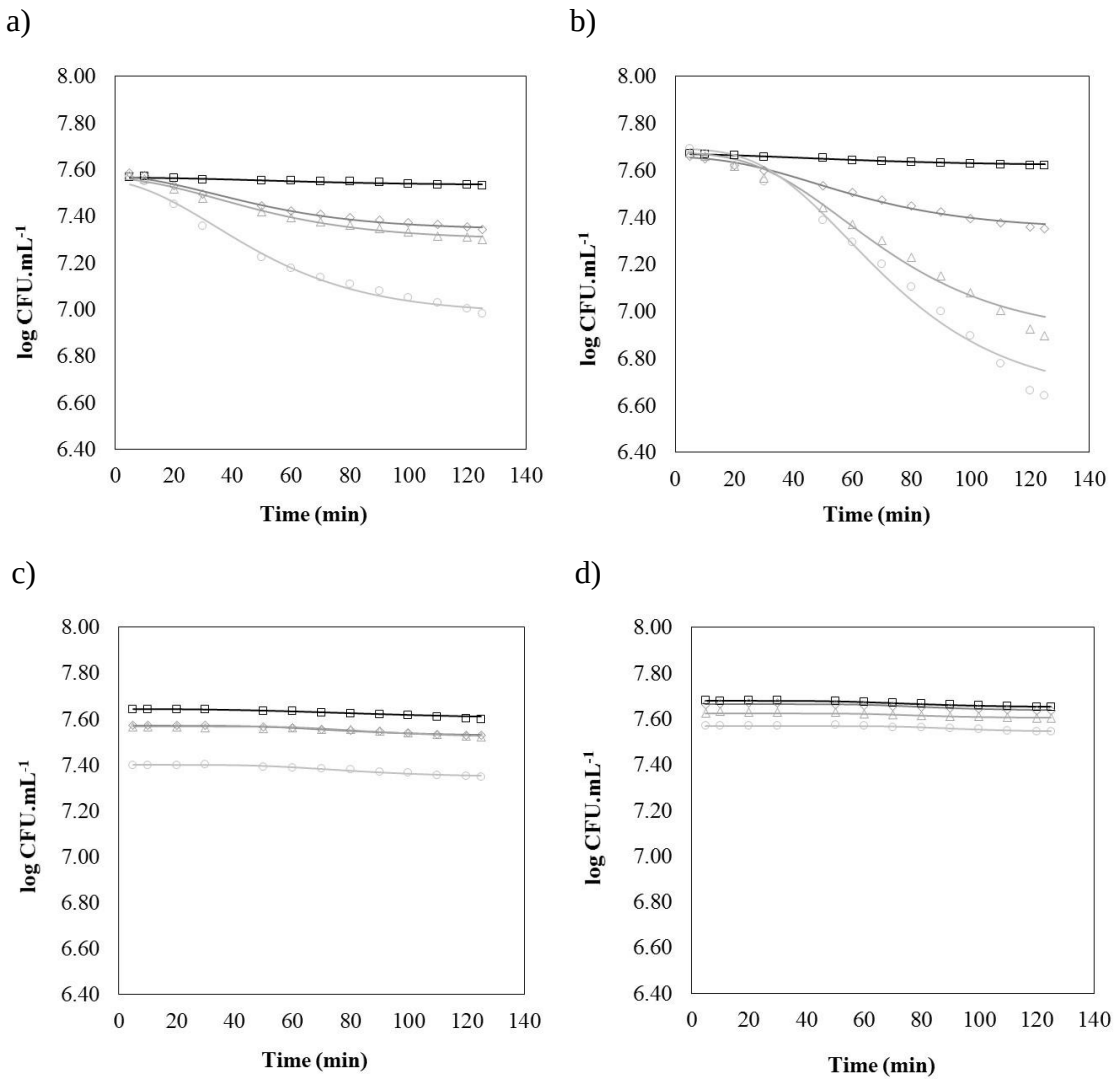


**Figure 4.5.** Time kill curves of SH (a), NEOW (b), CD (c) and NaDCC (d) at four different concentrations (20, 50, 80 and 100 ppm). The lines represent the Gompertz models for the experimental data. To facilitate the observation, not all the points measured are represented. SH – sodium hypochlorite; NEOW – neutral electrolyzed oxidizing water; CD – chlorine dioxide; NaDCC - sodium dichloroisocyanurate; □ 20 ppm; ◊ 50 ppm; Δ 80 ppm; ○ 100 ppm; — 20 ppm model; — 50 ppm model; — 80 ppm model; — 100 ppm model.

**Table 4.3.** Kinetic parameters of the Gompertz model for the experimental data obtained in the time kill curves, for the four biocides (SH, NEOW, CD and NaDCC) at four different concentrations (20, 50, 80 and 100 ppm). $k_{CFU}$ is the kinetic constant (log CFU.mL$^{-1}$.min$^{-1}$) that represents the maximum antimicrobial rate, $\lambda_{CFU}$ is the time for antimicrobial action (min), $R^2$ is the coefficient of determination obtained with the Gompertz model and RMSE is the root mean square error (log CFU.mL$^{-1}$)

| Biocide | Concentration (ppm) | $k_{CFU}$ (log CFU.mL$^{-1}$.min$^{-1}$) | $\lambda_{CFU}$ (min) | $R^2$ | RMSE (log CFU.mL$^{-1}$) |
|---|---|---|---|---|---|
| SH | 20 | 0.027 | 13.077 | 0.932 | 0.030 |
| | 50 | 0.034 | 2.471 | 0.988 | 0.082 |
| | 80 | 0.033 | 3.316 | 0.988 | 0.093 |
| | 100 | 0.030 | 7.508 | 0.988 | 0.200 |
| NEOW | 20 | 0.032 | 15.315 | 0.980 | 0.024 |
| | 50 | 0.030 | 22.854 | 0.991 | 0.101 |
| | 80 | 0.031 | 25.075 | 0.985 | 0.316 |
| | 100 | 0.038 | 9.573 | 0.979 | 0.517 |
| CD | 20 | 0.025 | 34.683 | 0.916 | 0.190 |
| | 50 | 0.042 | 46.473 | 0.980 | 0.008 |
| | 80 | 0.046 | 58.887 | 0.954 | 0.012 |
| | 100 | 0.041 | 47.405 | 0.971 | 0.011 |
| NaDCC | 20 | 0.041 | 48.075 | 0.982 | 0.015 |
| | 50 | 0.051 | 59.778 | 0.911 | 0.037 |
| | 80 | 0.056 | 58.282 | 0.898 | 0.034 |
| | 100 | 0.061 | 72.069 | 0.935 | 0.025 |

(120 min, 80 and 100 ppm), the log CFU.mL$^{-1}$ reduction obtained was 0.274 and 0.591 for SH; 0.045 and 0.050 for CD; 0.021 and 0.025 for NaDCC, for 80 and 100 ppm, respectively. Those values are significantly different ($P < 0.05$) from those obtained with NEOW. Therefore, 80 ppm NEOW had a higher antimicrobial activity than 100 ppm SH, as it was able to promote higher log CFU.mL$^{-1}$ reduction (1.3 times higher). While, NaDCC was the least effective biocide in eliminating *E. coli*. SH demonstrated to be the fastest biocide having antimicrobial action. NaDCC and CD had the highest antimicrobial rates. When NEOW was used, the highest log CFU.mL$^{-1}$ reductions were achieved, which means that cell growth was controlled in a higher extent by NEOW, although the antimicrobial rate was lower than when using the other biocides.

### 4.2.3. Reactive oxygen species

The formation of ROS was assessed in order to understand the distinct antimicrobial effects of the biocides tested (Figure 4.6). ROS, such as singlet oxygen, hydrogen

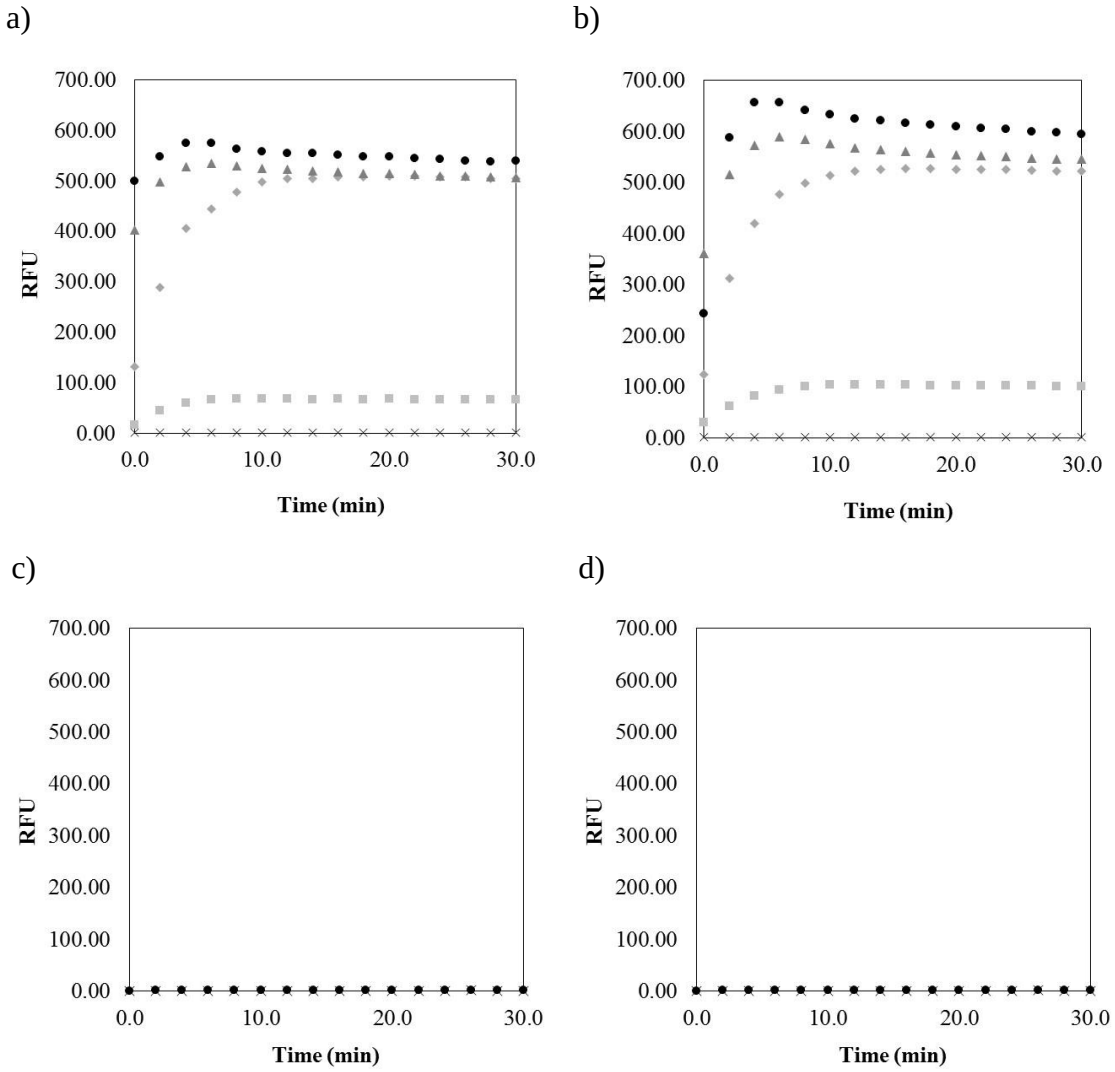


**Figure 4.6.** Relative fluorescence units (RFU) for SH (a), NEOW (b), CD (c) and NaDCC (d) at four different concentrations (20, 50, 80 and 100 ppm) and the control (cells with DCFH-DA). SH – sodium hypochlorite; NEOW – neutral electrolyzed oxidizing water; CD – chlorine dioxide; NaDCC - sodium dichloroisocyanurate; × Control; ■ 20 ppm; ♦ 50 ppm; ▲ 80 ppm; ● 100 ppm.

peroxide, superoxide and hydroxyl radical, are normally produced during cell metabolism (Jambunathan, 2010). However, in some conditions, such as the addition of certain biocides, ROS formation increases exponentially destroying cells' structure and function (Cabiscol et al., 2000). However, only biocides that have oxygen to react with the cells, producing a superoxide anion that is a precursor and propagator of oxidative chain reactions, promote ROS production (Turrens, 2003, Rastogi et al., 2010). In this study, only SH and NEOW lead to the production of ROS (Figure 4.6). No relative fluorescence units (RFU) were detected with the use of CD and NaDCC, proposing that under the conditions tested those biocides do not induce ROS production. Additionally, when comparing the RFU after SH and NEOW treatments, the maximum values obtained for

each condition tested were: 68.00 (20 ppm), 510.00 (50 ppm), 534.00 (80 ppm), 575.00 (100 ppm) for SH and 103.50 (20 ppm), 526.50 (50 ppm), 590.00 (80 ppm), 657.00 (100 ppm) for NEOW. In fact, the RFU values were always higher for NEOW ($P < 0.05$), which justifies the highest ability of this biocide to produce ROS and helps to explain the highest antimicrobial effects of NEOW.

### 4.2.4. Chemical stability of chlorine-based solutions

The chemical stability of the biocides, in terms of chlorine depletion rate, was measured overtime (Figure 4.7), allowing the assessment of kinetic parameters (Table 4.4).

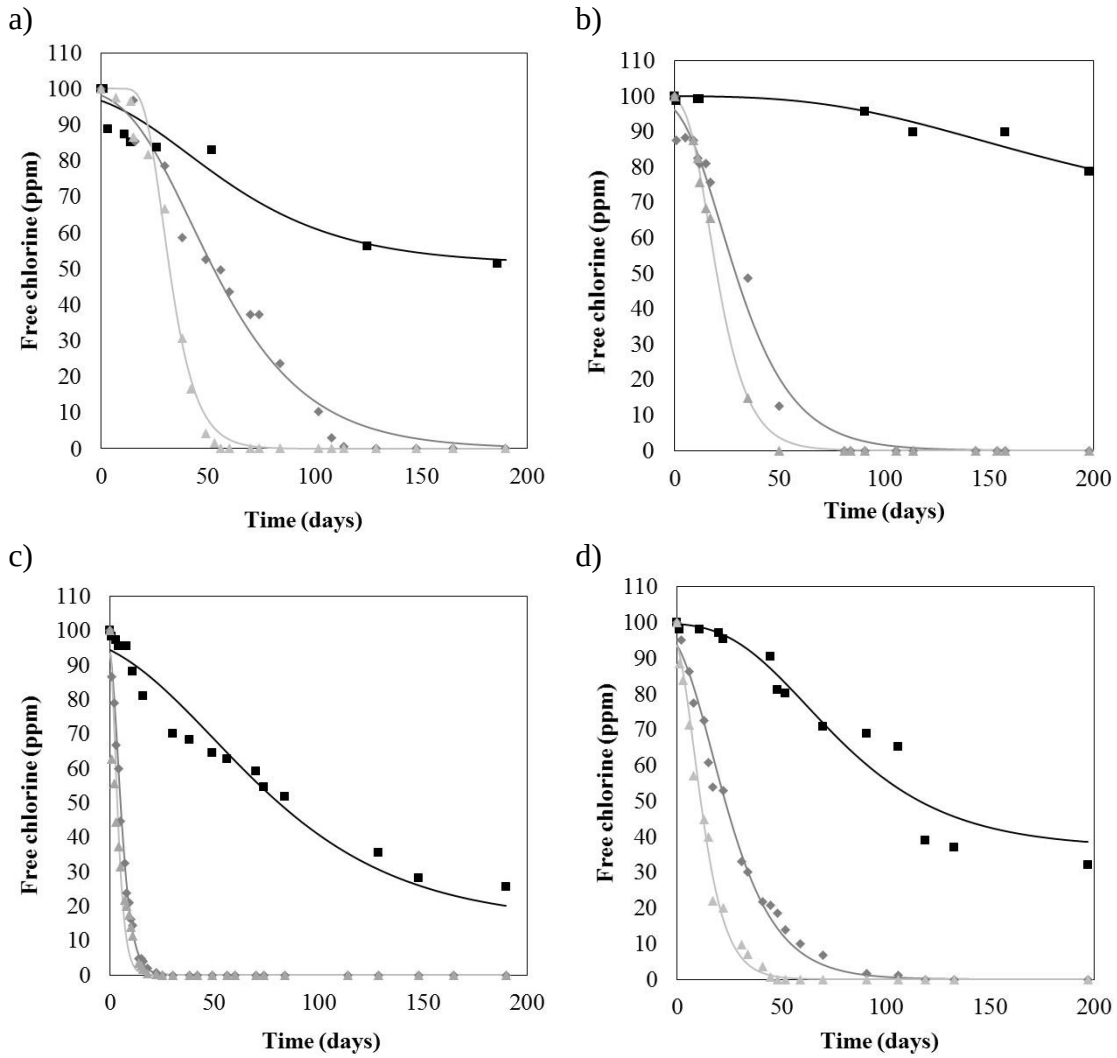


**Figure 4.7.** Chemical stability of SH (a), NEOW (b), CD (c) and NaDCC (d) at three different temperatures (5, 25 and 30 ºC). The lines represent the Gompertz models for the experimental data. SH – sodium hypochlorite; NEOW – neutral electrolyzed oxidizing water; CD – chlorine dioxide; NaDCC - sodium dichloroisocyanurate; ■ 5 °C; ♦ 25 °C; ▲ 30 °C; — 5 °C model; — 25 °C model; — 30 °C model.

From the model curves, the $R^2$ (close to 1) and RMSE values, the Gompertz model demonstrated to be suitable for describing the biocides decay. When comparing the decay time for free chlorine loss, at 5 ºC, NEOW was the most stable biocide with a depletion time of 70 days ($P < 0.05$), while for NaDCC the depletion time was 25 days and for SH and CD it was 0 days. In what concerns chlorine loss rate, at 5 ºC SH and NaDCC had the highest value (0.025 and 0.027 ppm.min$^{-1}$, respectively). It has been published that NaDCC antimicrobial action and effectiveness is similar to SH (Heling et al., 2001). At 5 ºC, NEOW has the lowest chlorine loss rate ($P < 0.05$), proving that this is a more stable biocide, with a lower chlorine loss. This is an important feature in the food industry, since most disinfection procedures are performed at temperatures around 5 ºC (Parish et al., 2003) and the maintenance of a stable and effective biocide concentration is fundamental to control microbial contaminations. Temperatures of 25 and 30 °C were also studied since they are commonly used in laboratory studies to test biocides efficacy (Kim et al., 2001, Borges et al., 2012, Abreu et al., 2014). At 25 °C SH demonstrated to be more stable than NEOW, CD and NaDCC, with a delay time for chlorine loss of 12 days, which is higher than all the other biocides ($P < 0.05$). At the same time the chlorine loss rate value for SH was inferior having a value of 0.033 ppm.min$^{-1}$. CD was the biocide with higher chlorine loss rate with $k_{FC}$ values of 0.281 ppm.min$^{-1}$ ($P < 0.05$), followed by NaDCC and NEOW that had $k_{FC}$ values of 0.060 and 0.053 ppm.min$^{-1}$ ($P > 0.05$), respectively. At 30 ºC SH had once again a higher delay time for chlorine loss (20 days). However, NEOW had the smallest $k_{FC}$ value (0.098 ppm.min$^{-1}$) and it was followed by SH (0.117 ppm.min$^{-1}$), NaDCC (0.123 ppm.min$^{-1}$) and CD with the highest chlorine loss rate (0.414 ppm.min$^{-1}$). Additionally, among all the biocides, CD had the fastest chlorine loss (0 days) for all temperatures tested ($P < 0.05$).

### 4.2.2. Tests with sessile cells in an agitated reactor

Further tests were performed by exposing sessile cells to the biocides at 50 ppm for 20 min (Figure 4.8). The log CFU.cm$^{-2}$ reductions were 3.26, 3.20, 2.64 and 2.46 for NEOW, CD, NaDCC and SH, respectively. These results show that NEOW and CD caused higher biofilm CFU.cm$^{-2}$ reductions than SH and NADCC ($P > 0.05$). Previous studies also demonstrated the high efficacy of NEOW and CD on the control of sessile cells. Arevalos-Sánchez et al. (2013) applied 70 ppm NEOW for 3 min against 4 days old *Listeria monocytogenes* biofilms developed on SS surfaces causing 2 log CFU.cm$^{-2}$ reduction.

**Table 4.4.** Kinetic parameters of the Gompertz model for the experimental data obtained in the chemical stability evaluation, for the four biocides (SH, NEOW, CD and NaDCC) at three different temperatures (5, 25 and 30 ºC). $k_{FC}$ is the kinetic constant (ppm.days$^{-1}$) that represents the maximum chlorine loss rate, $\lambda_{FC}$ is the time for chlorine loss (days), $R^2$ is the coefficient of determination obtained with the Gompertz model and RMSE is the root mean square error (ppm)

| Biocide | Temperature (ºC) | $k_{FC}$ (ppm.days$^{-1}$) | $\lambda_{FC}$ (days) | $R^2$ | RMSE (ppm) |
|---|---|---|---|---|---|
| SH | 5 | 0.025 | 0.000 | 0.886 | 15.077 |
| | 25 | 0.033 | 11.829 | 0.976 | 20.531 |
| | 30 | 0.117 | 20.219 | 0.986 | 19.484 |
| NEOW | 5 | 0.013 | 70.342 | 0.935 | 8.548 |
| | 25 | 0.053 | 3.606 | 0.991 | 16.746 |
| | 30 | 0.098 | 6.337 | 0.998 | 6.308 |
| CD | 5 | 0.020 | 0.000 | 0.957 | 19.597 |
| | 25 | 0.281 | 0.000 | 0.998 | 6.094 |
| | 30 | 0.414 | 0.000 | 0.874 | 34.849 |
| NaDCC | 5 | 0.027 | 24.785 | 0.955 | 21.891 |
| | 25 | 0.060 | 0.000 | 0.988 | 14.408 |
| | 30 | 0.123 | 0.000 | 0.990 | 12.953 |

These authors also used the same conditions (concentration and time) to test SH and obtained the same log reduction, concluding that NEOW and SH had similar effects on the control of SS-adhered *L. monocytogenes*. Kim et al. (2001) used 56 ppm NEOW to control 2 days old *L. monocytogenes* biofilms formed on SS surfaces. These authors observed a reduction of 9 log CFU.cm$^{-2}$ after 5 min exposure. Kreske et al. (2006) used 200 ppm CD against *B. cereus* biofilms formed on SS coupons causing 4.42 log CFU reduction. Robbins et al. (2005) used CD at 50 000 ppm for 10 min and obtained 4.14 log CFU.chip$^{-1}$ reduction of *L. monocytogenes* biofilms. These authors used higher concentrations than the one tested in this study. This helps to explain the higher CFU reductions obtained. The differences in the NEOW and CD biofilm CFU reduction between previous reports and the results of the present study are apparently related to the different bacterial species/strain selected for biofilm formation and control, as well as to the distinct process conditions (biofilm age, adhesion surface, biocide exposure time). Regarding the biofilm control action of NADCC and SH, previous studies propose a lower biofilm control action when compared to NEOW or CD. Block (2004) used 1000 ppm NaDCC for 10 min to remove *Clostridium difficile* and *Bacillus atrophaeus* from SS surfaces and obtained reductions of 1 and 1.5 log CFU, respectively. Ungurs et al. (2011) obtained similar results: 2.19 log CFU reduction of *C. difficile* biofilms from

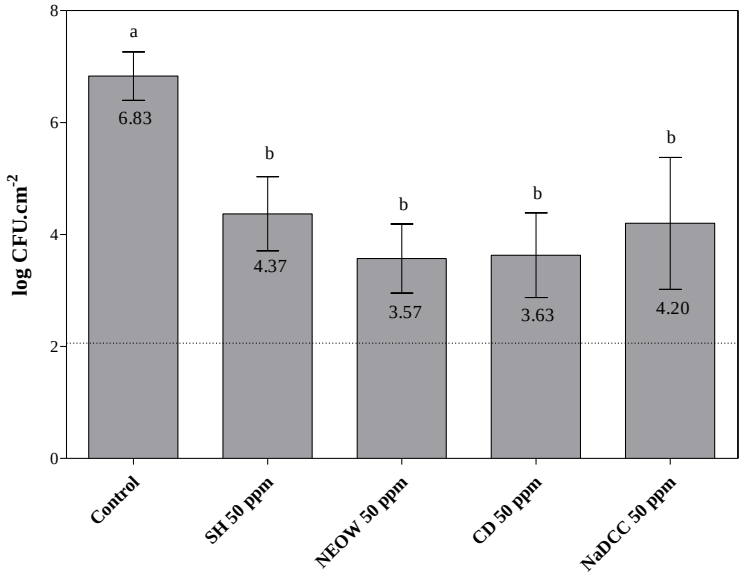


**Figure 4.8.** Log CFU.cm$^{-2}$ achieved after the application of the four biocides at 50 ppm. The line indicates the method detection limit (2.06 log CFU.cm$^{-2}$). Different letters represent statistically different values ($P < 0.05$). The error bars represent the standard deviation. SH – sodium hypochlorite; NEOW – neutral electrolyzed oxidizing water; CD – chlorine dioxide; NaDCC - sodium dichloroisocyanurate.

SS surfaces when applying 1000 ppm NaDCC for 20 min. Regarding SH, Kim et al. (2016) applied 50 ppm SH for 1 min on SS and achieved only 0.34 log CFU.cm$^{-2}$ reduction of *L. monocytogenes*. Rossoni and Gaylarde (2000) have also used SH to remove *E coli* biofilm from SS surfaces and were able to eliminate 1.77 log cells.cm$^{-2}$ using 100 ppm SH for 10 min.

From the planktonic tests, SH was the fastest biocide, NaDCC exhibited the higher antimicrobial rates, CD and NaDCC had a similar antimicrobial activity and NEOW had the highest antimicrobial action. The ROS determination proposed that antimicrobial actions of NEOW and SH were related to ROS formation. In the biofilm tests, NEOW and CD were the biocides that allowed higher log CFU reduction (3.26 and 3.20 log CFU.cm-2 for NEOW and CD, respectively). Therefore, NEOW demonstrated to be more effective than SH in the control of both planktonic and sessile bacteria. Furthermore, in the food industry, the fresh produce is typically disinfected at temperatures around 5 ºC, thus the stability at this temperature is important. NEOW had a longer decay time for chlorine loss (70 days) and the smallest chlorine loss rate (0.013 ppm.min$^{-1}$) at 5 ºC. Consequently, the overall results propose that SH can be replaced by a greener alternative

(NEOW) that is more effective in microbial growth control and has lower over time chlorine decay. In addition and according to the literature (Demirci and Bialka, 2010), no organochlorinated by-products from the use of NEOW are produced.

## 4.3. Characterization of the heterotrophic bacteria isolated from a MPV plant

The consumption of MPV or RTE vegetables has increased alongside with the consequent increase on illness outbreaks associated to human pathogens (Foong-Cunningham et al., 2012). Therefore, microbial identification and characterization of the food microflora is crucial to control food safety. This work aimed to understand the biodiversity and dynamics of microbial contamination in a MPV processing plant. Consequently, in this study the isolates were characterized for virulence factors production and susceptibility to antibiotics, to collect information about their potential public health concern. Their survival in a stomach mimicking environment was also studied. Additionally, autoaggregation and coaggregation were assessed to understand how the isolates interact in multispecies communities. Moreover, as first the alternative disinfectants were tested for *E. coli* CECT 434, it was important to understand the microorganisms' diversity present in the MPV in order to direct the disinfection to the more resilient microorganisms. Consequently, biofilm removal, killing and regrowth were studied for two disinfectants: SH, as it is the disinfectant of choice and also PA, since it was defined as a good alternative biocide in section 4.1.

### 4.3.1. Bacteria isolation and identification

This work enabled the disclosure and characterization of heterotrophic bacteria present in a MPV plant. From the sampling, 47 distinct bacteria were isolated: 18 were obtained from the air (16 from the washing room and 2 from the high care area), 6 were isolated from the produce leaves (3 from the washing room and 3 from the high care area) and 23 were collected from the food-contact surfaces (10 from the washing room and 13 from the high care area). With the sampling performed and the sampling points chosen it is not possible to guarantee that these are the only microorganisms present in the MPV plant. From the samples taken after the sanitization procedure with Tego 2000® it was not possible to isolate any microorganism, proposing that sanitization was effective in microbial growth control. The isolation method used allowed further identification of 20 different bacterial species (identity percentage never lower than 98%) being 49%

*Pseudomonas* spp. The review of Siroli et al. (2015) also describes *Pseudomonas* spp. as the predominant microbial population in minimally processed fruits and vegetables. These authors also mentioned the predominant presence of *Enterobacteriaceae*, such as *Erwinia* spp. and *Rahnella aquatilis*. In the present study about 11% of the isolates were *Enterobacteriaceae*. With the RNA gene sequencing sequences, a phylogenetic tree was constructed (Figure 4.9) and one can conclude from the high bootstrap values that the

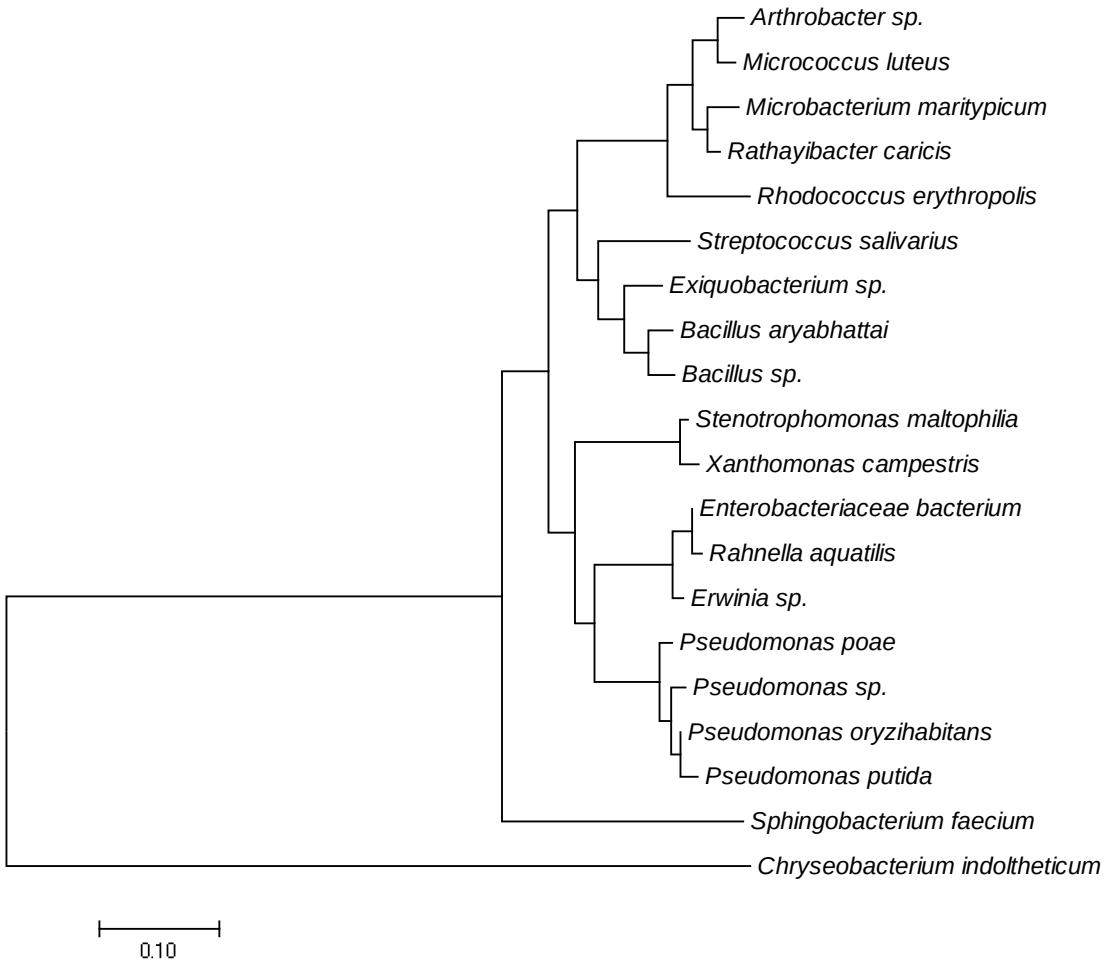


**Figure 4.9.** Phylogenetic tree for the isolated bacteria from the MPV plant. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 1.39 is shown. The percentages of replicate in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the p-distance method (Nei and Kumar, 2000) and are in the units of the number of base differences per site. The analysis involved 20 nucleotide sequences. Codon positions included were $1^{st}+2^{nd}+3^{rd}$+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 367 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013).

results are statistically significant and indicate support for a clade (Soltis and Soltis, 2003). From Figure 4.9 it is possible to observe seven distinct branches: 1) *Arthobacter* sp., *Micrococcus luteus*, *Microbacterium maritypicum*, *Rathayibacter caricis* and *Rhodococcus erythropolis* (phylum and class of Actinobacteria); 2) *Streptococcus salivarius*, *Exiguobacterium* sp., *Bacillus aryabhattai* and *Bacillus* sp. (phylum Firmicutes and class Bacilli); 3) *Stenotrophomonas maltophilia* and *Xanthomonas campestris* (phylum Proteobacteria and class Gammaproteobacteria); 4) *Enterobacteriaceae bacterium*, *Rahnella aquatilis* and *Erwinia* sp. (phylum Proteobacteria, class Gammaproteobacteria and order Enterobacteriales); 5) *Pseudomonas poae*, *Pseudomonas* sp., *P. oryzihabitans* and *P. putida* (phylum Proteobacteria, class Gammaproteobacteria, order Pseudomonadales); 6) *Sphingobacterium faecium* (phylum Bacteroidetes and class Sphingobacteria) and 7) *Chryseobacterium indoltheticum* (phylum Bacteroidetes and class Flavobacteria).

After bacterial isolation and identification, it was possible to infer on the possible microbial presence in the process. Observing the results in Table 4.5, *Pseudomonas* sp. was present in the contaminated product at the beginning of the decontamination process and it was also in the two tanks of the washing room, in the air and, more critically, in the high care area (conveyor belt) and in the final product. Furthermore, *P. poae* and *R. aquatilis* were also present in the final product and in previous steps of the process (washing tank). This means that the produce decontamination using 70-90 ppm of chlorine was able to reduce the bacteria present but was not 100% efficient in eliminating *Pseudomonas* sp. from the process. Furthermore, it was not possible to identify the exact source of this contamination, as it was generically dispersed (in the initial product, in the washing room, in the high care area and in the air). Moreover, *P. poae* and *R. aquatilis* were also isolated from the washing tank and remained in the high care area, particularly in the conveyor belt after optical sorting and in the final product. The source of contamination was not identified but could be the water used to wash the produce. In fact, *R. aquatilis* natural habitat is water (Harrell et al., 1989). Moreover, it can be proposed that *Pseudomonas* sp., *P. poae* and *R. aquatilis* coexist in the biofilm, as they were isolated in the same critical points. It is possible that when these microorganisms are in community they form a resilient biofilm (Flemming et al., 2016). Additionally, *P. oryzihabitans* appears to be the main bacteria contaminating the air in the washing

**Table 4.5.** Microorganisms isolated from several critical points in the MPV plant (surfaces - tanks and conveyor belts, produce, and air). The numbers correspond to the sampling points represented in Figure 3.1. Microorganisms marked in bold are the persisting ones

| Area | Zone | Source | Microorganism |
|---|---|---|---|
| Washing room | ① Washing tank | Surface 1 | ***R. aquatilis*** |
| | | | ***Pseudomonas* sp.** |
| | | | ***P. poae*** |
| | | | *E. bacterium* |
| | | Surface 2 | *B. aryabhattai* |
| | | | ***Pseudomonas* sp.** |
| | | Leaves | *Arthrobacter* sp. |
| | | | *Erwinia* sp. |
| | | | ***Pseudomonas* sp.** |
| | ② Sanitizing tank | Surface 1 | ***Pseudomonas* sp.** |
| | | Surface 2 | - |
| | | Surface 3 | - |
| | ③ Air | | *C. indoltheticum* |
| | | | *S. faecium* |
| | | | *P. putida* |
| | | | *Bacillus* sp. |
| | | | *M. maritypicum* |
| | | | *R. caricis* |
| | | | ***Pseudomonas* sp.** |
| | | | *S. salivarius* |
| | | | ***P. oryzihabitans*** |
| High care | ④ Waste water tank | Surface | - |
| | ⑤ Conveyor belt | Surface 1 | - |
| | before dryer | Surface 2 | - |
| | ⑥ Conveyor belt after optical sorting | Surface 1 | ***R. aquatilis*** |
| | | | ***P. poae*** |
| | | | ***Pseudomonas* sp.** |
| | | | ***P. oryzihabitans*** |
| | | Surface 2 | *Pseudomonas* sp. |
| | | | *X. campestris* |
| | | Surface 3 | *S. maltophilia* |
| | | | *Exiguobacterium* sp. |
| | | | *R. erythropolis* |
| | | Leaves | ***P. poae*** |
| | | | ***R. aquatilis*** |
| | | | ***Pseudomonas* sp.** |
| | ⑦ Air | | ***Pseudomonas* sp.** |
| | | | *M. luteus* |

room, possibly being transferred to the high care area - to the conveyor belt after optical sorting. Therefore, the results propose that *Pseudomonas* sp., *P. poae*, *R. aquatilis* and

also *P. oryzihabitans* are apparently the bacteria tolerating decontamination, predominating along the process chain and being present in the final produce. However, those bacteria were susceptible to Tego 2000® during the sanitization step.

Even if no reference foodborne pathogen was isolated, some of the bacteria are of potential public health concern, such as *P. oryzihabitans*, *S. maltophilia* and *R. aquatilis*. *P. oryzihabitans* has been described as pathogenic for infants (Freney et al., 1988). Several authors (Denton and Kerr, 1998, Di Bonaventura et al., 2004, Pompilio et al., 2008, Pompilio et al., 2011, Brooke, 2012) reported that *S. maltophilia* is a pathogenic microorganism, considered as emerging multidrug resistant. *R. aquatilis* has been linked to human disease in rare instances, most commonly in immunosuppressed individuals (Gaitan and Bronze, 2010). Despite the microbial pathogenic potential, the spoilage action is also important (Madigan et al., 2009). In fact, some of the isolated bacteria have already been described as causes of vegetable spoilage: *X. campestris* (Liao, 2006), *Pseudomonas* spp. and *Erwinia* spp. (Tournas, 2005).

### 4.3.2. Bacteria characterization

*4.3.2.1. Quorum sensing inhibition and virulence factors production*

The results on QSI, siderophores, gelatinases and proteases production are present in Table 4.6. As can be observed *P. putida*, *Pseudomonas* sp. and *E. bacterium* were the only bacteria able to inhibit QS. Some microorganisms are able to inhibit QS and consequently control biofilm formation of neighbor bacteria (McLean et al., 2004). This can be an indication of microbial predominance and prevalence in the environment. Some pathogenic microorganisms use AI such as AHLs to mediate QS (Borges et al., 2014). These molecules are synthetized by AHL synthethases (LuxI family) that can bind to transcription regulators from the LuxR family (Greenberg, 2000). In this study, the determination of QSI was performed with *C. violaceum* CV12472. This bioreporter strain produces and responds to the AIs C6-AHL and C4-AHL (Borges et al., 2014). *P. putida*, *Pseudomonas* sp. and *E. bacterium* were the only bacteria able to inhibit QS. To study AHL production strains *C. violaceum* ATCC 31532 and *A. tumefaciens* KYC6 were used as positive controls. *C. violaceum* ATCC 31532 can produce C4-HSL (*N*-butanoyl homoserine lactone) and C6-HSL (*N*-hexanoyl homoserine lactone), while *A. tumefaciens* KYC6 can produce AHL C6-C14. The results demonstrated that

**Table 4.6.** QSI and production of siderophores, gelatinases and proteases by the microorganisms isolated from the MPV plant. n.g. - no growth; shaded cells - tests not performed due to the negative QSI

| Bacteria | QSI | C4-HSL and C6-HSL | AHL C6 - C14 | Siderophores | Proteases | Gelatinases |
|---|---|---|---|---|---|---|
| *M. luteus* | - | | | n.g. | + (11.0 mm) | + (2.0 mm) |
| *S. faecium* | - | | | n.g. | - | - |
| *P. putida* | + | - | - | + (5.5 mm) | + (9.0 mm) | + (2.0 mm) |
| *Bacillus* sp. | - | | | n.g. | + (15.0 mm) | + (9.0 mm) |
| *P. oryzihabitans* | - | | | + (5.0 mm) | - | - |
| *R. caricis* | - | | | + (0.5 mm) | + (3.5 mm) | + (4.0 mm) |
| *S. salivarius* | - | | | n.g. | + (5.0 mm) | - |
| *M. maritypicum* | - | | | n.g. | - | + (4.0 mm) |
| *Pseudomonas* sp. | + | - | - | + (1.0 mm) | - | + (4.0 mm) |
| *E. bacterium* | + | + | + | + (5.0 mm) | - | - |
| *C. indoltheticum* | - | | | - | + (11.0 mm) | + (2.5 mm) |
| *P. poae* | - | | | + (1.2 mm) | - | - |
| *R. aquatilis* | - | | | + (1.0 mm) | - | - |
| *Arthrobacter* sp. | - | | | n.g. | + (11.0 mm) | + (4.0 mm) |
| *Erwinia sp.* | - | | | + (1.0 mm) | - | - |
| *X. campestris* | - | | | + (0.5 mm) | + (10.0 mm) | - |
| *S. maltophilia* | - | | | - | + (11.0 mm) | + (5.0 mm) |
| *R. erythropolis* | - | | | + (0.8 mm) | - | - |
| *Exiguobacterium* sp. | - | | | + (0.5 mm) | + (17.0 mm) | + (11.0 mm) |
| *B. aryabhattai* | - | | | + (3.0 mm) | + (9.0 mm) | + (6.5 mm) |

*E. bacterium* produced the same type of molecules that were produced by *C. violaceum* ATCC 31532 and *A. tumefaciens* KYC6. The molecules produced by *P. putida* and *Pseudomonas* sp. were not identified by the method used and additional tests with other biosensors should be performed. However, these results allow to propose that *P. putida*, *Pseudomonas* sp. and *E. bacterium* can potentially avoid biofilm formation from the other microorganisms, encouraging their own persistence on a surface. Examples of these visual tests are present in Figure 4.10 (a, b, c and d).

Siderophores are chelating agents that scavenge iron and make it available to the microbial cells and these molecules are produced by the microorganisms when they are in an environment with iron limitations (Neilands, 1995). The production of siderophores can be considered harmful when the microorganisms are present in human hosts, as a

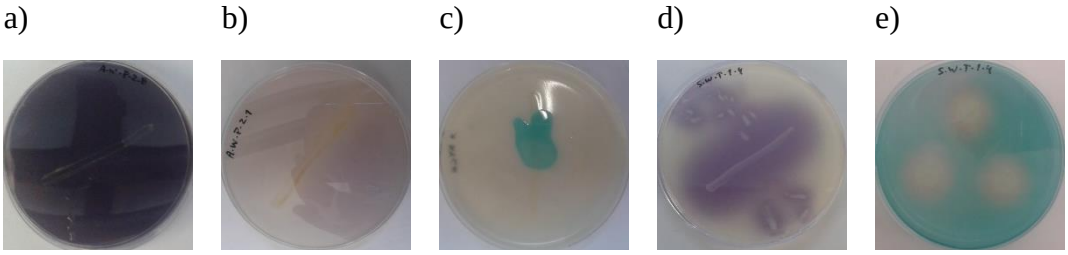


**Figure 4.10.** Visual QSI results: a) example of a bacterium (*M. maritypicum*) that did not inhibit QS from *C. violaceum* ATCC 12472; b) example of a bacterium (*P. putida*) that inhibited QS from *C. violaceum* ATCC 12472; c) example of a bacterium (*E. bacterium***)** that can produce C4-HSL and C6-HSL; d) example of a bacterium (*E. bacterium***)** that can produce AHL C6-C14; e) example of a bacterium (*E. bacterium*) that can produce siderophores.

certain iron level must be maintained ($10^{-24}$ mol.$L^{-1}$) in the human body (Raymond et al., 2003). From the isolated bacteria, *P. putida* was the one with the highest siderophores production ability as evidenced by the halo diameter (Table 4.6). *C. indoltheticum* and *S. maltophilia* were not able to produce siderophores, while *M. luteus*, *S. faecium*, *Bacillus* sp., *S. salivarius*, *M. maritypicum* and *Arthrobacter* sp. were not able to grow in the CAS agar plates. According to Raymond et al. (2003) microorganisms able to produce siderophores are potentially pathogenic. In fact, some of the isolates producing siderophores have already been described as pathogenic, particularly *P. oryzihabitans* (Freney et al., 1988) and *R. aquatilis* (Flores-Tena et al., 2007). In Figure 4.10e an example of the visual test can be observed.

Proteases and gelatinases are enzymes that degrade proteins and are produced by the microorganisms to obtain amino acids for microbial growth. They are both considered virulence aspects and can act on the host tissues (Lopes et al., 2006, Costa et al., 2010). Proteases can hydrolyse hemoglobin and keratin (Costa et al., 2010) and gelatinases can hydrolyse gelatin, collagen, fibrinogen, casein, insulin and hemoglobin (Kayaoglu and Ørstavik, 2004, Lopes et al., 2006). *Exiguobacterium* sp. is protease positive, reinforcing the findings of Kasana and Yadav (2007), and was the microorganism with the highest protease activity (halo of 17.0 mm). *S. faecium*, *P. oryzihabitans*, *M. maritypicum*, *Pseudomonas* sp., *E. bacterium*, *P. poae*, *R. aquatilis*, *Erwinia* sp. and *R. erythopolis* were unable to produce proteases. *Bacillus* sp., *M. luteus*, *C. indoltheticum*, *Arthrobacter* sp., *S. maltophilia* and *X. campestris* were good protease producers. Clark et al. (2000) also found protease producing strains of *Bacillus* sp. and *M. luteus*, as well as Brooke (2012) found for *C. indoltheticum*, *Arthrobacter* sp. and *S. maltophilia.*

Regarding gelatinase production, 45% of the bacteria were not able to produce gelatinases: *S. faecium*, *P. oryzihabitans*, *S. salivarius*, *E. bacterium*, *P. poae*, *R. aquatilis*, *Erwinia* sp., *X. campestris* and *R. erythropolis*. *S. faecium* was described as gelatinase negative by Takeuchi and Yokota (1992) as well as *R. aquatilis* by Chang et al. (1999) *Exiguobacterium* sp. was the microorganism that produced more gelatinases (halo of 11.0 mm). *S. maltophilia* strains were also described as gelatinase positive by Brooke (2012), as well as *B. aryabhattai* by Ray et al. (2012), and *M. maritypicum* was already described as gelatinase positive by Takeuchi and Hatano (1998).

The only microorganism that was able to inhibit QS and produce all the virulence factors was *P. putida*. Additionally, *P. oryzihabitans* was one of the bacteria that produced more siderophores, not producing any of the other molecules tested; *S. salivarius* only produced proteases. *S. faecium* was the only microorganism that did not express any of the molecules analyzed for the conditions used in this study.

*4.3.2.2. Antibiotic susceptibility*

The results obtained are present in Table 4.7. As the isolated bacteria are not described in the CLSI (2005) guidelines, *E. coli* and *S. aureus* were used as controls. In general, all Gram-positive isolates were susceptible to tetracycline, erythromycin, levofloxacin and ciprofloxacin. Tetracycline inhibits the synthesis of proteins preventing bacterial growth (Chopra and Roberts, 2001). Erythromycin is a macrolide that inhibits the synthesis of proteins leading to cell death (Vardanyan and Hruby, 2016). Levofloxacin and ciprofloxacin are both quinolones and interfere with DNA replication and transcription (Davies, 2013). All Gram-positive isolates are resistant to ampicillin, except *Exiguobacterium* sp., which is susceptible ($P < 0.05$). Ampicillin is a β-lactam antibiotic and its mode of action is based on the inhibition of the bacteria cell wall synthesis (Briñas et al., 2002). However, most microorganisms are resistant to this antibiotic due to their ability to produce β-lactamases (Vardanyan and Hruby, 2016). Additionally, it should be pointed out that the inhibition zone diameter of ampicillin is zero for *M. maritypicum*, *R. caricis* and *R. erythropolis*. Comparing with *S. aureus*, it is possible to observe that the results of the isolates and this specific Gram-positive bacterium are very similar (all resistant and susceptible to the same antibiotics).

For the Gram-negative isolates they are all susceptible to levofloxacin and ciprofloxacin. However, they are all resistant to ampicillin (inhibition zone diameter of zero).

**Table 4.7.** Antimicrobial activity of antibiotics on the isolates from the RTE vegetables industry. Marked in a shaded color are the controls (*E. coli* CECT 434 and *S. aureus* CECT 976). R – resistant; I – intermediate; S - susceptible

| **Microorganism** | **Gram** | **Diameter of inhibition zone (cm)** | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | **Tetracycline** | | **Erythromycin** | | **Ampicillin** | | **Levofloxacin** | | **Ciprofloxacin** | |
| *S. aureus* CECT 976 | + | 2.6 ± 0.1 | S | 3.4 ± 0.1 | S | 1.8 ± 0.1 | R | 3.9 ± 0.1 | S | 3.9 ± 0.1 | S |
| *Arthrobacter* sp. | + | 2.4 ± 0.3 | S | 3.4 ± 0.1 | S | 1.3 ± 0.2 | R | 2.4 ± 0.1 | S | 2.4 ± 0.1 | S |
| *B. aryabhattai* | + | 2.9 ± 0.2 | S | 3.4 ± 0.2 | S | 1.6 ± 0.0 | R | 4.0 ± 0.2 | S | 3.8 ± 0.3 | S |
| *Bacillus* sp. | + | 2.8 ± 0.2 | S | 2.9 ± 0.1 | S | 1.9 ± 0.2 | R | 3.5 ± 0.1 | S | 3.2 ± 1.0 | S |
| *Exiguobacterium* sp. | + | 2.6 ± 0.2 | S | 3.0 ± 0.1 | S | 3.1 ± 0.3 | S | 2.6 ± 0.2 | S | 2.5 ± 0.2 | S |
| *M. maritypicum* | + | 2.2 ± 0.2 | S | 5.3 ± 0.3 | S | 0.0 ± 0.0 | R | 2.6 ± 0.3 | S | 2.7 ± 0.2 | S |
| *M. luteus* | + | 3.6 ± 0.2 | S | 3.6 ± 0.1 | S | 1.3 ± 0.2 | R | 2.6 ± 0.0 | S | 2.9 ± 0.2 | S |
| *R. caricis* | + | 4.2 ± 0.4 | S | 5.7 ± 0.4 | S | 0.0 ± 0.0 | R | 3.8 ± 0.2 | S | 3.5 ± 0.1 | S |
| *R. erythropolis* | + | 2.1 ± 0.2 | S | 2.3 ± 0.2 | S | 0.0 ± 0.0 | R | 3.2 ± 0.1 | S | 2.9 ± 0.4 | S |
| *S. salivarius* | + | 3.1 ± 0.1 | S | 3.6 ± 0.3 | S | 1.4 ± 0.8 | R | 2.4 ± 0.2 | S | 2.5 ± 0.2 | S |
| *E. coli* CECT 434 | - | 1.1 ± 0.0 | R | 1.2 ± 0.1 | - | 0.0 ± 0.0 | R | 3.7 ± 0.1 | S | 3.8 ± 0.1 | S |
| *C. indoltheticum* | - | 1.7 ± 0.3 | I | 2.2 ± 0.2 | I | 0.0 ± 0.0 | R | 3.2 ± 0.2 | S | 3.1 ± 0.3 | S |
| *E. bacterium* | - | 1.3 ± 0.2 | R | 1.4 ± 0.2 | I | 0.0 ± 0.0 | R | 2.8 ± 0.2 | S | 2.7 ± 0.1 | S |
| *Erwinia* sp. | - | 2.8 ± 0.2 | S | 1.3 ± 0.1 | R | 0.0 ± 0.0 | R | 3.4 ± 0.2 | S | 4.0 ± 0.5 | S |
| *P. oryzihabitans* | - | 1.9 ± 0.3 | S | 0.0 ± 0.0 | R | 0.0 ± 0.0 | R | 2.7 ± 0.4 | S | 3.0 ± 0.5 | S |
| *P. poae* | - | 2.4 ± 0.1 | S | 1.5 ± 0.2 | I | 0.0 ± 0.0 | R | 3.4 ± 0.1 | S | 3.6 ± 0.2 | S |
| *P. putida* | - | 1.9 ± 0.2 | S | 0.0 ± 0.0 | R | 0.0 ± 0.0 | R | 3.1 ± 0.1 | S | 3.6 ± 0.4 | S |
| *Pseudomonas* sp. | - | 2.9 ± 0.0 | S | 1.7 ± 0.0 | I | 0.0 ± 0.0 | R | 3.3 ± 0.2 | S | 3.2 ± 0.1 | S |
| *R. aquatilis* | - | 1.3 ± 0.2 | R | 1.4 ± 0.2 | I | 0.0 ± 0.0 | R | 2.9 ± 0.2 | S | 3.2 ± 0.2 | S |
| *S. faecium* | - | 1.8 ± 0.1 | I | 2.0 ± 0.4 | I | 0.0 ± 0.0 | R | 2.9 ± 0.1 | S | 2.8 ± 0.1 | S |
| *S. maltophilia* | - | 0.9 ± 0.0 | R | 0.0 ± 0.0 | R | 0.0 ± 0.0 | R | 3.4 ± 0.3 | S | 2.8 ± 0.2 | S |
| *X. campestris* | - | 3.6 ± 0.3 | S | 3.2 ± 0.2 | S | 0.0 ± 0.0 | R | 4.5 ± 0.6 | S | 4.2 ± 0.5 | S |

Values are presented as the mean (cm) ± standard deviation for at least three independent experiments.

*E. bacterium*, *R. aquatilis* and *S. maltophilia* are resistant to tetracycline and *C. indoltheticum* and *S. faecium* are classified as intermediate. Erythromycin is usually applied against Gram-positive bacteria (Davies, 2013), as the Gram-negative bacteria outer membrane prevents the entering of hydrophobic compounds such as macrolides (Saha et al., 2008). However, in this study it was decided to apply this antibiotic against the Gram-negative bacteria. It was found that *X. campestris* was susceptible ($P < 0.05$) while *Erwinia* sp., *P. oryzihabitans*, *P. putida* and *S. maltophilia* were resistant. The control strain - *E. coli* – was also resistant to two antibiotics tested (tetracycline and ampicillin), which are the same antibiotics that *E. bacterium* and *R. aquatilis* were resistant. Additionally, 30% of the isolates (*E. bacterium*, *Erwinia* sp., *P. oryzihabitans*, *P. putida*, *R. aquatilis* and *S. maltophilia*), which are Gram-negative bacteria, were resistant to more than one antibiotic tested. Since the antibiotics are from different classes these isolates can be considered multidrug resistant (Davies and Davies, 2010). It has to be highlighted that *S. maltophilia* was the only bacteria with resistance to three antibiotics: tetracycline, erythromycin and ampicillin. It can also be observed that Gram-negative bacteria are more resistant to the antibiotics studied than the Gram-positive isolates.

*4.3.2.3. Aggregation*

The isolates were also tested for their aggregation potential in order to understand the interactions in specific biofilm microcosms. It has been described that the interspecies relationships and aggregation potential can contribute to biofilm formation (Simões et al., 2008). The results obtained (Table 4.8) show that only *R. erythopolis* was able to form aggregates. *R. erythropolis* was previously described to be able to produce a bioflocculant, helping to explain the autoaggregation ability (Kurane et al., 1986, Kurane et al., 1994). This property can be seen as an advantage for bacteria since it provides protection from adverse environmental conditions facilitating biofilm formation (Takeda et al., 1991, Kragh et al., 2016). Since *R. erythropolis* was the only isolate that was able to autoaggregate it was combined with the bacteria that were isolated from the same critical point in the RTE vegetables plant (conveyor belt after optical sorting in the high care area): *Exiguobacterium* sp., *P. oryzihabitans*, *P. poae*, *Pseudomonas* sp., *R. aquatilis*, *S. maltophilia*, *X. campestris* (Table 4.9). From these combinations and through the applied methods *R. erythropolis* did not seem to coaggregate with the bacteria that were isolated from the same critical point.

**Table 4.8.** Autoaggregation index and score for the isolates from the RTE vegetables industry

| Microorganism | Aggregation index (%) | Aggregation score | | | |
|---|---|---|---|---|---|
| | | 0 h | 2 h | 24 h | 48 h |
| *Arthrobacter* sp. | 2.6 ± 0.4 | 0 | 0 | 0 | 0 |
| *Bacillus aryabhattai* | 9.7 ± 1.0 | 0 | 0 | 0 | 0 |
| *Bacillus* sp. | 10.3 ± 2.6 | 0 | 0 | 0 | 0 |
| *Exiguobacterium* sp. | 3.5 ± 1.3 | 0 | 0 | 0 | 0 |
| *M. maritypicum* | 4.30 ± 0.8 | 0 | 0 | 0 | 0 |
| *M. luteus* | 13.2 ± 3.3 | 0 | 0 | 0 | 0 |
| *R. caricis* | 13.2 ± 0.2 | 0 | 0 | 0 | 0 |
| *R. erythropolis* | 56.4 ± 0.6 | 2 | 2 | 2/3 | 2/3 |
| *S. salivarius* | 4.1 ± 2.0 | 0 | 0 | 0 | 0 |
| *C. indoltheticum* | 1.4 ± 0.7 | 0 | 0 | 0 | 0 |
| *E. bacterium* | 7.2 ± 2.4 | 0 | 0 | 0 | 0 |
| *Erwinia* sp. | 4.4 ± 1.8 | 0 | 0 | 0 | 0 |
| *P. oryzihabitans* | 28.2 ± 2.1 | 0 | 0 | 0 | 0 |
| *P. poae* | 5.7 ± 1.7 | 0 | 0 | 0 | 0 |
| *P. putida* | 14.8 ± 0.2 | 0 | 0 | 0 | 0 |
| *Pseudomonas* sp. | 3.6 ± 0.2 | 0 | 0 | 0 | 0 |
| *R. aquatilis* | 1.4 ± 0.0 | 0 | 0 | 0 | 0 |
| *S. faecium* | 3.6 ± 1.0 | 0 | 0 | 0 | 0 |
| *S. maltophilia* | 3.2 ± 0.9 | 0 | 0 | 0 | 0 |
| *X. campestris* | 7.1 ± 0.4 | 0 | 0 | 0 | 0 |

Values are presented as the mean (%) ± standard deviation for at least three independent experiments.

#### *4.3.2.4. Stomach environment survival*

The consumption of fresh produce has been considered as the highest concern group for microbiological safety, as the RTE vegetables are consumed raw with reduced disinfection (Mritunjay and Kumar, 2015). Since the impact on public health is an important factor to consider, the isolates obtained from the RTE vegetables industry were studied for their persistence if ingested. Therefore, all the isolates were tested in a simulated gastric environment. From the tests using the simulated stomach, none of the bacteria survived to the conditions tested. Nevertheless, with this simulation only the healthy individuals were considered, since the conditions used are based on a healthy person (Guerra et al., 2012). However, if an individual with a weak or suppressed immune system ingests RTE vegetables that may contain these bacteria the outcome is unknown.

**Table 4.9.** Coaggregation index and score for *R. erythropolis* combined with the bacteria isolated from the same place in the RTE vegetables industry

| Microorganism | Coaggregation index (%) | Coaggregation score | | | |
|---|---|---|---|---|---|
| | | 0 h | 2 h | 24 h | 48 h |
| *Exiguobacterium* sp. | 17.3 ± 2.5 | 0 | 0 | 0 | 0 |
| *P. oryzihabitans* | 39.1 ± 9.7 | 1 | 1 | 1 | 1 |
| *P. poae* | 27.0 ± 0.4 | 0 | 0 | 0 | 0 |
| *Pseudomonas* sp. | 22.9 ± 1.4 | 0 | 0 | 0 | 0 |
| *R. aquatilis* | 28.3 ± 1.8 | 0 | 0 | 0 | 0 |
| *S. maltophilia* | 39.0 ± 8.6 | 1 | 1 | 1 | 1 |
| *X. campestris* | 6.8 ± 2.0 | 0 | 0 | 0 | 0 |

Values are presented as the mean (%) ± standard deviation for at least three independent experiments.

*4.3.2.5. Biofilm formation in 96-well PS plates*

All the bacteria were capable of forming biofilms with different productivities (Figure 4.11). *P. oryzihabitans* and *S. salivarius* were moderate biofilm formers and all the other bacteria were weak biofilm producers. From all the isolates *P. oryzihabitans* was the microorganism that formed more biofilm ($P < 0.05$) and *B. aryabhattai* was the microorganism that produced less biofilm ($P < 0.05$). *Pseudomonas* spp. are typically recognized as biofilm formers (Toutain et al., 2004, Mann and Wozniak, 2012). Pompilio et al. (2008) have also described that *S. maltophilia* can form biofilms. Neverthless, all the isolated bacteria are biofilm producers, even if with different productivities.

*4.3.2.6. Biofilm formation on SS, removal, elimination and regrowth*

SH is the typically disinfectant used in the MPV industry (Ramos et al., 2013). However its application leads to the production of mutagenic and carcinogenic organochlorinated by-products when in contact with organic matter (Bull et al., 2011). Thus, alternative disinfectants have been tested and described as more advantageous (section 4.1). From the several possible alternatives, PA was selected to test the bactericidal action against the isolated bacteria since it was considered a good alternative in section 4.1. Therefore, the effects of SH and PA were tested on biofilm removal (Figure 4.12) and killing (Figure 4.13). *P. oryzihabitans* produced denser biofilms on the SS surface (6.72 ± 0.27 log cells.cm$^{-2}$) ($P > 0.05$ for *Bacillus* sp., *R. erythropolis*, *S. salivarius*, *E. bacterium*, *Erwinia* sp., *P. putida* and *X. campestris*), while *Exiguobacterium* sp. and *B. aryabhattai* were the isolates that formed less biofilm (5.08 ± 0.21 and 5.08 ± 0.48 log cells.cm$^{-2}$,

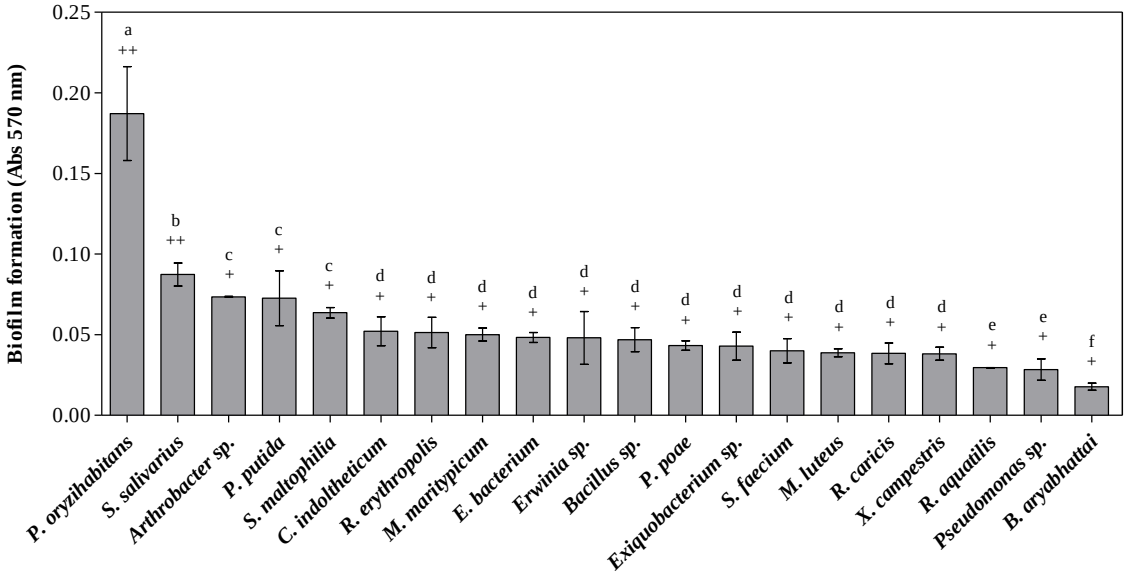


**Figure 4.11.** Biofilm formation (absorbance at λ = 570 nm) for the bacteria isolated from the MPV plant. Different letters represent statistically different values ($P < 0.05$). The error bars represent the standard deviation. + weak biofilm producers; + + moderate biofilm producers.

respectively). None of the disinfectants was efficient in biofilm removal. The log cells.cm$^{-2}$ reduction for both disinfectants was not statistically different from the control, for all 20 isolates ($P > 0.05$). The maximum reduction obtained for both disinfectants was against *Pseudomonas* sp. biofilms (0.44 and 0.35 log cells.cm$^{-2}$, for SH and PA respectively). For 55% of the isolates (*Exiguobacterium* sp., *M. maritypicum*, *M. luteus*, *R. caricis*, *R. erythropolis*, *S. salivarius*, *E. bacterium*, *P. oryzihabitans*, *P. poae*, *Pseudomonas* sp. and *S. faecium*) 50 ppm SH was more efficient than 50 ppm PA, even if not statistically significant ($P > 0.05$). For 35% of the isolates (*Arthrobacter* sp., *C. indoltheticum*, *Erwinia* sp., *P. putida*, *R. aquatilis*, *S. maltophilia* and *X. campestris*) PA was more efficient than SH ($P > 0.05$). PA and SH had no effects on the removal of *B. aryabhattai* and *Bacillus* sp. biofilms ($P > 0.05$).

From Figure 4.13 it is possible to observe that for *B. aryabhattai* and *Exiguobacterium* sp. it was not possible to detect CFU's for any of the conditions tested, due to the reduced ability of these bacteria to adhere or form biofilm on SS in a level above the detection limit of the method. The poor biofilm formation ability of these isolates has already been described in section 4.3.2.5. Additionally, *P. oryzihabitans* was the microorganism that formed more biofilm ($P < 0.05$ for *B. aryabhattai*, *Exiguobacterium* sp., *M. luteus*, *M. maritypicum*, *C. indoltheticum*, *P. poae* and *Pseudomonas* sp.). This result was

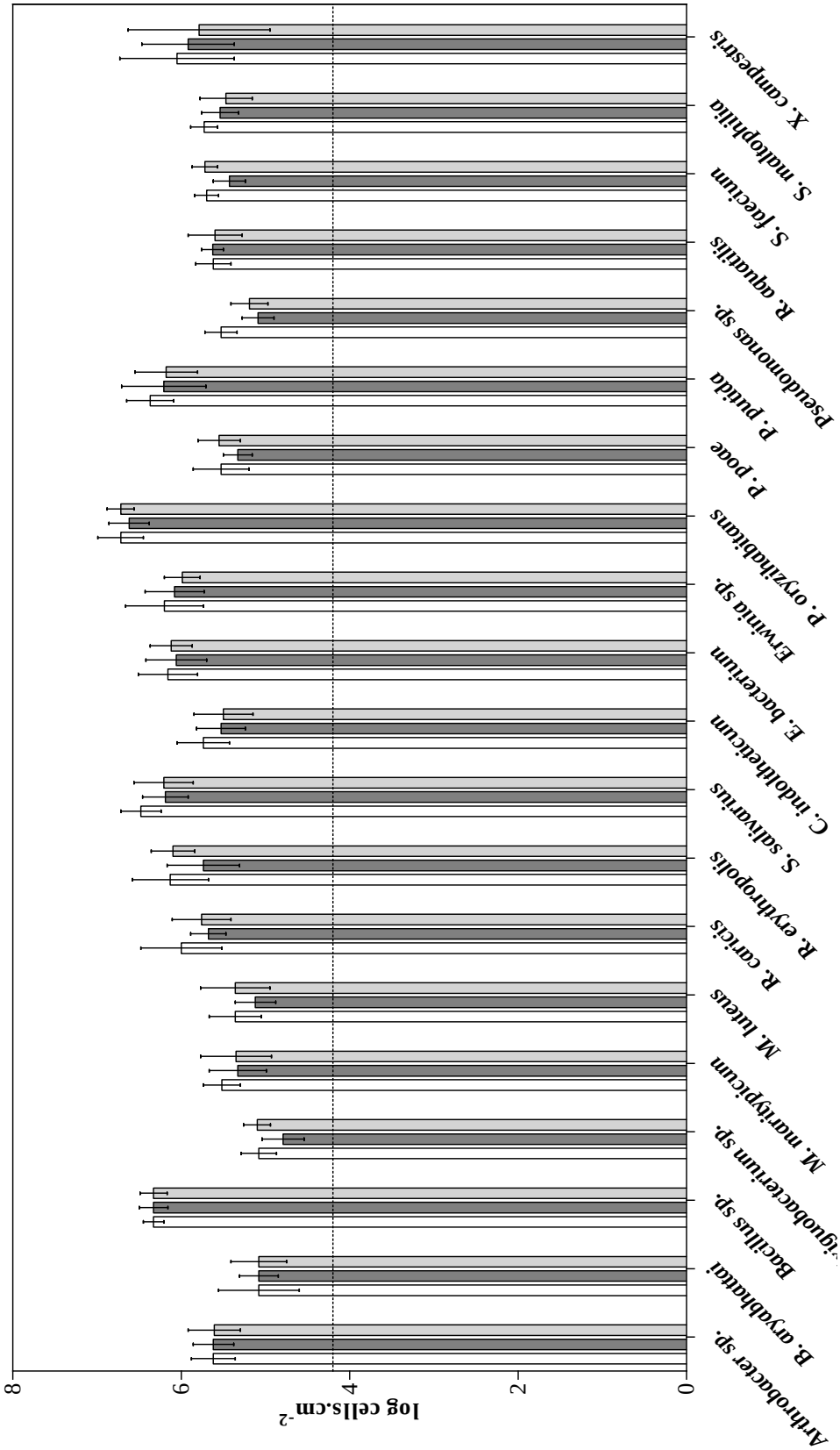


**Figure 4.12.** Log cells.cm$^{-2}$ removal achieved after the application of the individual disinfectants (SH 50 ppm and PA 50 ppm) against biofilms formed on SS. The line indicates the method detection limit (4.20 log cells.cm$^{-2}$). The error bars represent the standard deviation. □ Control; ■ SH 50 ppm; ■ PA 50 ppm.

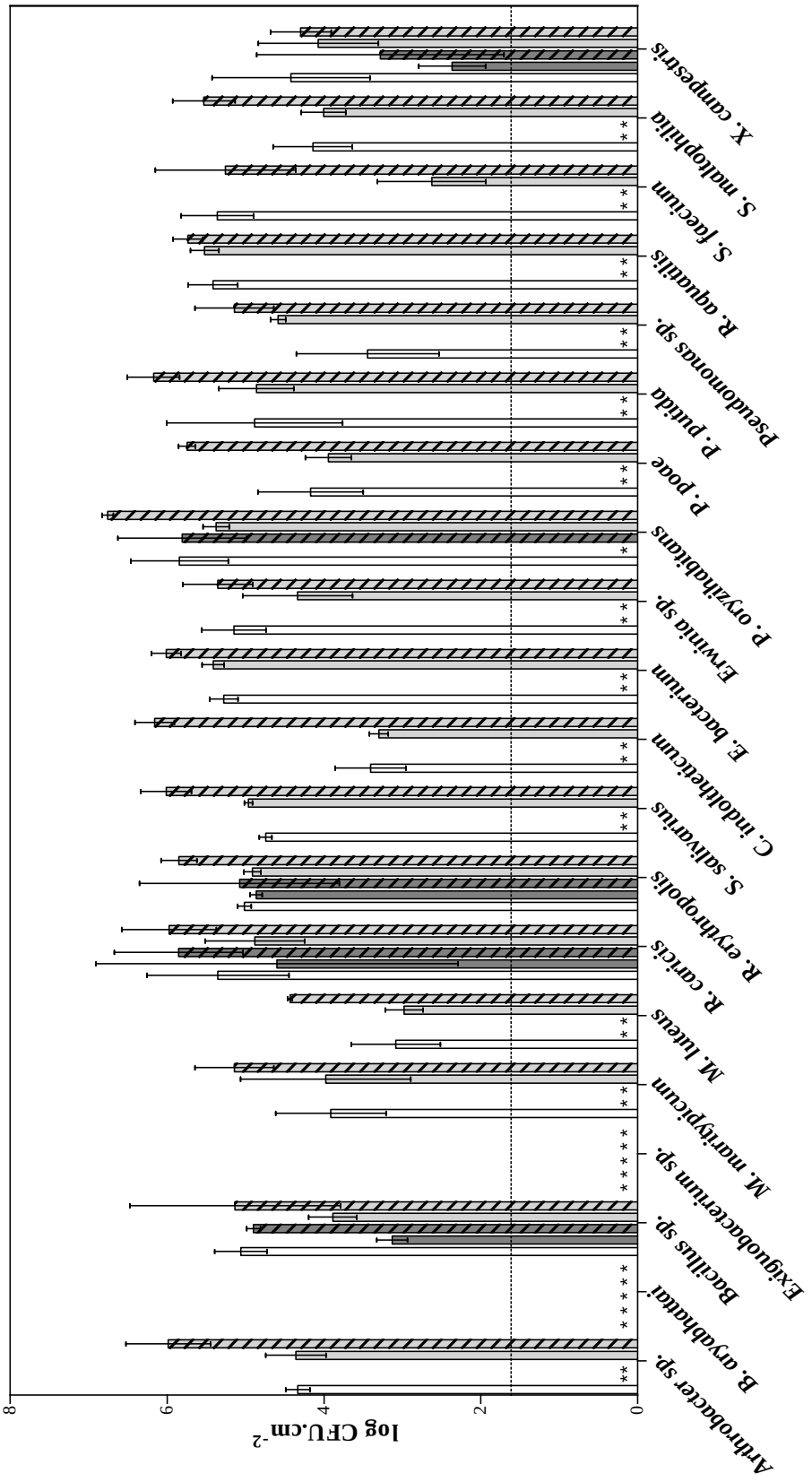


**Figure 4.13.** Log CFU.cm$^{-2}$ reduction of biofilms formed on SS. The individual disinfectants' action (SH 50 ppm and PA 50 ppm) is represented, as well as the regrowth phenomena. The line indicates the method detection limit (1.62 log CFU.cm$^{-2}$). The symbol * represents that no CFU was detected. The error bars represent the standard deviation. □ Control; ■ SH 50 ppm; ▬ SH 50 ppm regrowth; ■ PA 50 ppm; ▬ PA 50 ppm regrowth.

expected since *Pseudomonas* spp. are usually described as good biofilm formers (Toutain et al., 2004, Mann and Wozniak, 2012). About 70% of the isolates (*Arthrobacter* sp., *M. maritypicum*, *M. luteus*, *S. salivarius*, *C. indoltheticum*, *E. bacterium*, *Erwinia* sp., *P. oryzihabitans*, *P. poae*, *P. putida*, *Pseudomonas* sp., *R. aquatilis*, *S. faecium* and *S. maltophilia*) were completely killed using 50 ppm SH (no culturable cells detected). SH at 50 ppm was not effective in total CFU reduction for *Bacillus* sp., *X. campestris* (log CFU.cm$^{-2}$ reduction was statistically different from the control, $P < 0.05$), *R. caricis* and *R. erythropolis* (log CFU.cm$^{-2}$ reduction was not statistically different from the control, $P > 0.05$). All these isolates were able to regrow markedly after SH exposure ($P < 0.05$). This effect was particularly significant for *R. caricis* and *P. oryzihabitans*. For PA action, one can notice that this disinfectant was not able to cause total CFU reduction against the biofilms tested. The maximum effect was observed with *S. faecium* (2.73 log CFU.cm$^{-2}$ reduction; $P < 0.05$).

From all the cases where no CFU were detected, only *P. oryzihabitans* was able to regrow from SH exposure ($P > 0.05$). Which means that despite the fact that there were no CFU after SH 50 ppm application, there were still some viable but non-culturable cells that were able to grow once the new media was added. Therefore, the study of biofilm regrowth is important to assess the microorganisms' behavior against disinfectants. In other words, it is crucial to verify if the microorganisms are able to overcome disinfectant action and develop resistant biofilms. In fact, as bacteria were not completely removed (as seen in Figure 4.12) the regrowth phenomena occurred and the log CFU.cm$^{-2}$ increased significantly ($P < 0.05$) for *Arthrobacter* sp., *Bacillus* sp., *M. luteus*, *C. indoltheticum*, *P. oryzihabitans*, *P. poae*, *P. putida*, *S. faecium* and *S. maltophilia*. Consequently, neither PA or SH at 50 ppm demonstrated to be reliable to remove or kill the biofilms formed by the isolated bacteria. This can be detrimental in the cases where the suitable conditions are provided and the bacteria are able to recover and regrow. This was the case for every bacteria treated with PA and for some treated with SH (*Bacillus* sp., *P. oryzihabitans*, *R. caricis*, *R. erythropolis*, *X. campestris* and *R. erythropolis*).

The results showed that the predominant microflora present in the MPV were also the genera with the highest biofilm formation ability – *Pseudomonas*. Also, the conveyor belt in the high care area was a significant source of contamination. Microbial action of biocides was modest and neither of the disinfectants was capable of removing the biofilm

cells. The results demonstrated that improvements in decontamination are needed to efficiently control the microflora in the final product.

## 4.4. Behavior comparison between a reference strain and a MPV isolate

The present study started with *E. coli* CECT 434 behavior against biocides, however, the microflora from a MPV industry was isolated and characterized. Therefore, the purpose of this section was to compare a reference strain (*E. coli* CECT 434) with a food isolate, in terms of antimicrobial resistance, biofilm formation and virulence compounds production. The aim was to determine if the reference strain was appropriate to standardize disinfection strategies, or if an isolate should be tested instead. *P. oryzihabitans* was the isolate chosen as it was the bacteria that formed more biofilm, from all the isolates.

### 4.4.1. MIC and MBC comparison

Observing the results obtained for the MIC (Table 4.10), the concentration needed for SH and PA to have an inhibitory action against *E. coli* CECT 434 is similar ($P > 0.05$). The MIC obtained is different from previous published works. Holah et al. (2002) found that 50 ppm of SH were sufficient to obtain a 5 log CFU reduction of *E. coli*, which is a much lower concentration than the one used in this study. On the other hand, Mazzola et al. (2009) used SH 1129 ppm to obtain a 6 log CFU reduction of *E. coli*, which is a 2.5 times higher concentration than the minimum achieved in this study. For *P. oryzihabitans*, the SH and PA concentration needed to inhibit the cells is different ($P < 0.05$) for both disinfectants. The concentration is higher for SH, which means that it is necessary a higher SH concentration to inhibit *P. oryzihabitans*, when compared to the necessary PA

**Table 4.10.** MIC and MBC obtained for the individual disinfectants for *E. coli* CECT 434 and *P. oryzihabitans*

| Bacteria | Disinfectant | MIC (ppm) | MBC (ppm) |
|---|---|---|---|
| *E. coli CECT 434* | Sodium hypochlorite | 447 ± 74 [a] | 447 ± 74 [a] |
| | Peracetic acid | 456 ± 0 [a] | 532 ± 91 [a] |
| *P. oryzihabitans* | Sodium hypochlorite | 608 ± 0 [b] | 684 ± 76 [b] |
| | Peracetic acid | 456 ± 0 [a] | 456 ± 76 [a] |

Values are presented as the mean (ppm) ± standard deviation for at least three independent experiments. Different letters within the same column represent statistically different values ($P < 0.05$).

concentration. Comparing the results for both bacteria, *P. oryzihabitans* is more resistant to SH. A higher concentration (608 ppm) was needed to inhibit the food isolate ($P < 0.05$). It can be inferred that, as it is a food isolate, persistently exposed to SH, *P. oryzihabitans* has increased tolerance to this disinfectant. Moreover, when PA is used the results obtained are similar to the ones obtained for *E. coli* CECT 434. For the MBC, the conclusions that can be drawn are exactly the same: *P. oryzihabitans* is more resistant to SH than *E. coli* CECT 434 ($P < 0.05$).

### 4.4.2. Antibiotic susceptibility

Antibiotic susceptibility was also tested and both bacteria were resistant and/or susceptible to the same antibiotics (Table 4.11). However, *E. coli* CECT 434 was more resistant to tetracycline ($P < 0.05$). The resistance can be due to the presence of efflux pumps that pump tetracycline out of the cell (Davies, 2013). On the other hand, *P. oryzihabitans* was more resistant to levofloxacin and ciprofloxacin ($P < 0.05$). Both bacteria were resistant to ampicillin. This result was already expected since ampicillin is a β-lactam, which means that it inhibits the bacteria cell wall synthesis and almost every bacteria can produce β-lactamases that destroy the antibiotic molecule (Vardanyan and Hruby, 2016). Several authors had already described *E. coli* CECT 434 resistance to ampicillin, tetracycline, levofloxacin and ciprofloxacin (Sáenz et al., 2004, Olorunmola et al., 2013). However, there is no information on antibiotic susceptibility for *P. oryzihabitans*.

### 4.4.3. QSI and virulence factors production

Neither of the bacteria were able to inhibit QS (Table 4.12). The inhibition of QS can indicate microbial prevalence since this inhibition can control biofilm formation from other bacteria (McLean et al., 2004). From all the other virulence factors tested, both bacteria were capable of producing only siderophores. However, *P. oryzihabitans* produced at a higher extent ($P < 0.05$). Siderophores production can be harmful if the bacteria are present in the human body (Schwyn and Neilands, 1987, Raymond et al., 2003), since these molecules are chelating agents that make iron available for the bacteria (Neilands, 1995). Siderophores production has already been described for both bacteria studied (Sharma and Johri, 2003, Demir and Kaleli, 2004, Vagrali, 2009). Neither of the bacteria were capable of producing proteases and gelatinases.

**Table 4.11.** Antimicrobial activity of antibiotics on *E. coli* CECT 434 and *P. oryzihabitans*. TET – tetracycline; AMP – ampicillin; LEV – levofloxacin; CIP – ciprofloxacin; R – resistant; S - susceptible

| Microorganism | Diameter of inhibition zone (cm) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | TET | | AMP | | LEV | | CIP | |
| *E. coli* CECT 434 | 1.10 ± 0.05 [a] | R | 0.00 ± 0.00 [a] | R | 3.70 ± 0.14 [a] | S | 3.80 ± 0.14 [a] | S |
| *P. oryzihabitans* | 1.90 ± 0.28 [b] | S | 0.00 ± 0.00 [a] | R | 2.70 ± 0.39 [b] | S | 2.97 ± 0.46 [b] | S |

Values are presented as the mean (cm) ± standard deviation for at least three independent experiments. Different letters within the same column represent statistically different values ($P < 0.05$).

### 4.4.4. Biofilm formation

Biofilm formation was first evaluated by CV staining (Table 4.13) and from the results obtained *E. coli* CECT 434 was considered a moderate biofilm producer and *P. oryzihabitans* was considered a week biofilm producer ($P < 0.05$). However, CV staining is not a selective and precise method, since the dye binds to negatively charged molecules. This method counts live and dead cells, and also the EPS present in the biofilm matrix (Chiba et al., 1998, Peeters et al., 2008). Observing Figure 4.14 this difference in biofilm formation is not perceptible, since both bacteria can form biofilms and the CFU number is statistically similar ($P > 0.05$). The problems associated with CV staining can be a justification for the discrepancy between results. Additionally, CV staining was done in PS surfaces and in the reactor, the surfaces used were SS. Therefore, the difference in surface material can also be a reason for the divergent results.

In terms of disinfectants efficacy, the concentration tested (50 ppm) was chosen because in the food industry SH concentration usually applied varies at 70-90 ppm and the

**Table 4.12.** Production of QSI, siderophores, gelatinases and proteases by *E. coli* CECT 434 and *P. oryzihabitans*

| Bacteria | QSI | Siderophores | Proteases | Gelatinases |
|---|---|---|---|---|
| *E. coli* CECT 434 | - | + (1.0 ± 0.0 mm) [a] | - | - |
| *P. oryzihabitans* | - | + (5.0 ± 0.0 mm) [b] | - | - |

Values are presented as the mean (cm) ± standard deviation for at least three independent experiments. Different letters represent statistically different values ($P < 0.05$).

**Table 4.13.** Biofilm formation for *E. coli* CECT 434 and *P. oryzihabitans* in a specific medium evaluated by CV staining

| Bacteria | O.D. 570 nm | Classification |
|---|---|---|
| *E. coli* CECT 434 | 0.275 ± 0.059 [a] | + + |
| *P. oryzihabitans* | 0.138 ± 0.035 [b] | + |

Values are presented as the mean ± standard deviation for at least three independent experiments. Different letters represent statistically different values ($P < 0.05$). + weak biofilm producers; + + moderate biofilm producers

producers want consistently to decrease this concentration (Rico et al., 2007, Goodburn and Wallace, 2013). Therefore, to meet the MPV industry requirements, the disinfectants were tested at 50 ppm. Observing Figure 4.14, the results obtained for both bacteria are not statistically different from each other ($P > 0.05$). From both disinfectants, only SH had a considerable effect ($P < 0.05$), with a higher CFU reduction (2.46 and 2.44 log CFU.cm$^{-2}$ for *E. coli* CECT 434 and *P. oryzihabitans* respectively). This log reduction was not statistically different from one bacteria to the other ($P > 0.05$). On the other hand, PA concentration used was not sufficient to eliminate both bacteria studied. However, for *P oryzihabitans* the log CFU.cm$^{-2}$ reduction was lower than the one achieved for *E. coli* CECT 434 (0.48 log CFU.cm$^{-2}$ *vs* 1.22 log CFU.cm$^{-2}$) ($P > 0.05$). In the concentration tested the disinfectants applied demonstrated to be unsuitable for biofilm elimination for both bacteria. Moreover, it was not possible to distinguish the antimicrobial resistance in biofilm elimination for both bacteria.

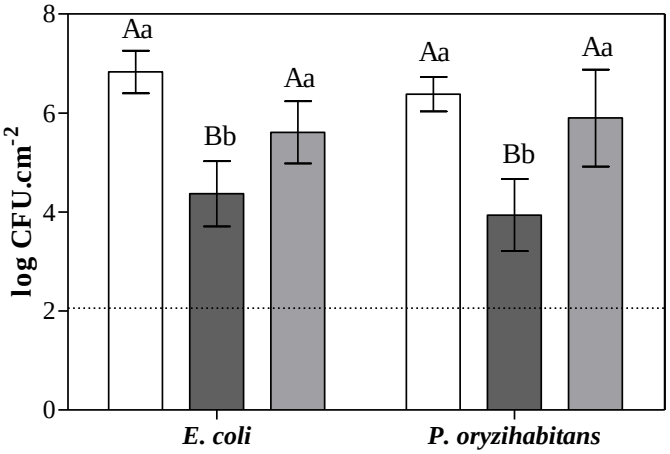


**Figure 4.14.** Log CFU.cm$^{-2}$ achieved after the application of SH and PA at 50 ppm for *E. coli* CECT 434 and *P. oryzihabitans*. The line indicates the method detection limit (2.06 log CFU.cm$^{-2}$). Different letters represent statistically different values within the same bacteria (A-C) and between both bacteria (a-c) ($P < 0.05$). The error bars represent the standard deviation. SH – sodium hypochlorite; PA – peracetic acid; □ Control; ■ SH 50 ppm; ■ PA 50 ppm.

To have a better understanding of the biofilm formation and structure the colonizing SS surfaces were observed by SEM (Figure 4.15). It is perceptible that *P. oryzihabitans* had a more thick and consistent biofilm that covers all the surface (Figure 4.15b). On the contrary, *E. coli* CECT 434 does not form such a uniform biofilm (Figure 4.15a). Moreover, the presence of EPS is more evident for *P. oryzihabitans* (Figure 4.15d). The results propose that the food isolate was more adapted to the cultivation conditions used, including a higher tendency to form biofilms on SS surfaces, which are usually used in the MPV industry, particularly in the washing and sanitizing tanks. Additionally, OCT

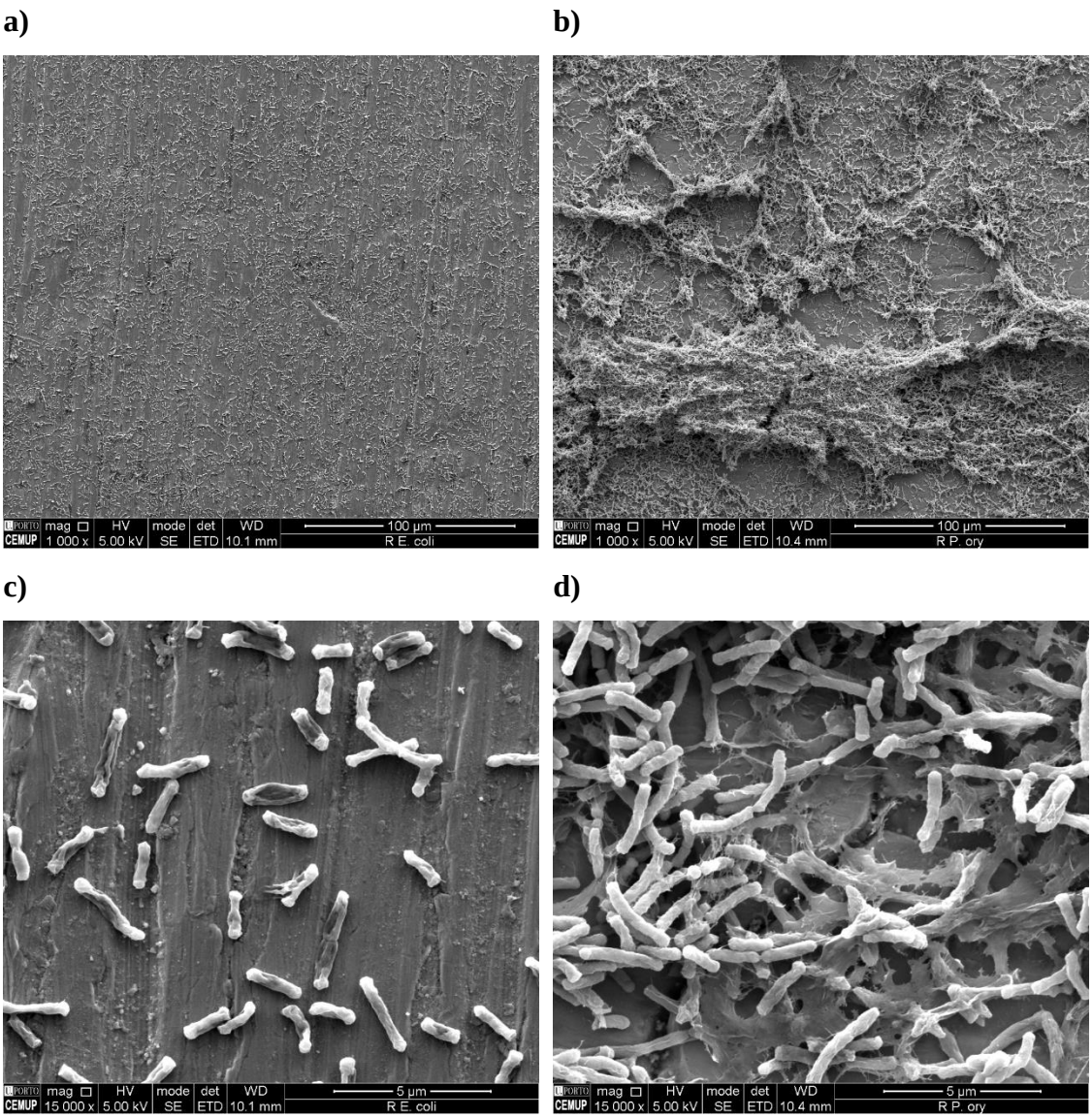


**Figure 4.15.** Scanning electron micrographs of *E. coli* CECT 434 (a and c) and *P. oryzihabitans* (b and d) 24-h biofilm on SS surfaces. Magnification: 1000× (a and b) (bars = 100 µm) and 15000× (c and d) (bars = 5 µm).

inspections (Figure 4.16) were performed to corroborate SEM results. Observing 5-day old biofilms formed on SS surfaces (Figure 4.16a), *E. coli* CECT 434 formed biofilms with a mushroom-like structure (Lawrence et al., 1991, de Beer et al., 1994, Klausen et al., 2003). *P. oryzihabitans* biofilms covered the surface more uniformly (Figure 4.16b).

*P. oryzihabitans* was considered more resistant to SH, PA and also to the tested antibiotics. This can be an indicative that isolates should be tested for alternative disinfectants, since the results for both bacteria were distinct.

## 4.5. Development of an artificial lettuce medium

The purpose of this work was to create a medium that would mimic fresh produce, allowing a future development of reliable disinfection strategies, with stronger suitability for further industrial application. To achieve this purpose the medium was simulated using the chemical composition of lettuces, proposed by Oke et al. (2012). Additionally, it was tested if the medium would promote antimicrobial tolerance, as it has been suggested that fresh produce is linked to a higher microbial resistance (Berger et al., 2010, Taban and Halkman, 2011, Schikora et al., 2012, Posada-Izquierdo et al., 2016). This new LTB medium was compared to MHB as this is the medium proposed by CLSI (2005) to test antimicrobial activity.

### 4.5.1. Growth study

The growth study (Figure 4.17) was done by $O.D._{600nm}$ measurements and CFU counting. It is possible to observe the four phases that are part of the growth curve: lag (0.0 – 2.0 h for MHB and 0.0 – 1.0 h for LTB), exponential (2.0 – 4.0 h for MHB and 1.0 – 4.0 h for LTB), stationary (4.0 – 9h for both media) and death phase (9.0 – 10.0 h for both media). In both media *E. coli* CECT 434 had a similar growth behavior but the lag phase was

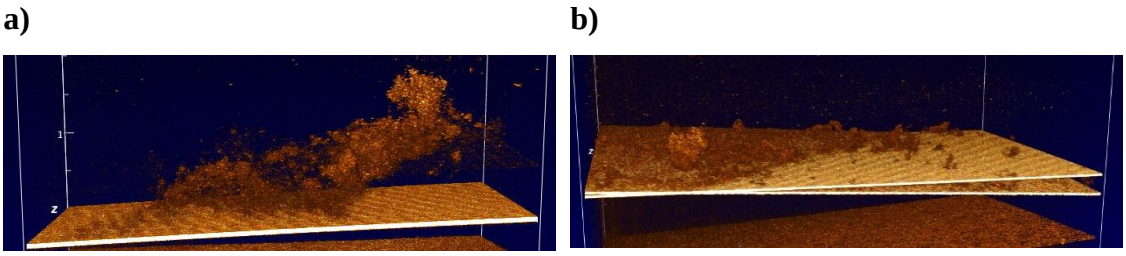


**Figure 4.16.** Optical coherence tomography of *E. coli* CECT 434 (a) and *P. oryzihabitans* (b) 5-day old biofilm on SS surfaces.

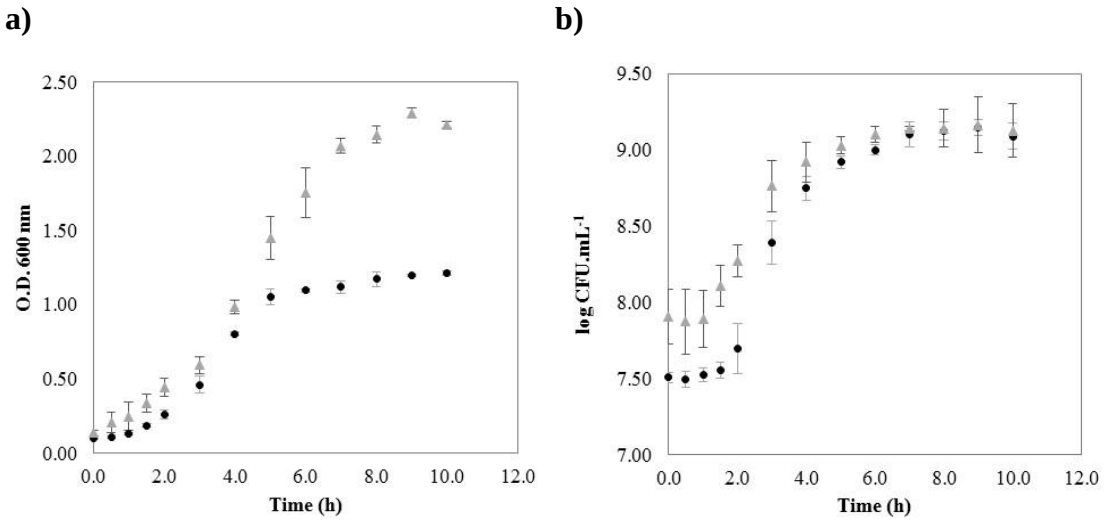


**Figure 4.17.** *E. coli* CECT 434 behavior in MHB and LTB. a) Growth curve with $O.D._{600nm}$ over time; b) Growth curve of CFU's over time. The error bars represent the standard deviation. ● MHB; ▲ LTB.

shorter in LTB, consequently the exponential phase was longer in LTB and the stationary and death phase had the same duration. Furthermore, observing Figure 4.17a it is possible to observe that the O.D. achieved with LTB was higher than the O.D. achieved with MHB. This difference is not the result of a higher log $CFU.mL^{-1}$. This is perceived from Figure 4.17b where the number of cells is similar in both media, not being statistically different from each other ($P > 0.05$). Therefore, the difference in O.D. can be due to diverse aspects: the microbial cells in different media can have different sizes; one medium can have more total cells, since spectrophotometry methods measure not only live cells but also dead cells (Pan et al., 2014); the microbial cells can be producing different components in the distinct media that affect the optical absorbance.

Regarding the growth rate calculated using the exponential phase, the value found was higher for MHB ($0.555\pm0.066$ $h^{-1}$) than for LTB ($0.444\pm0.160$ $h^{-1}$) ($P > 0.05$). This difference demonstrates that, despite the fact that the lag phase was longer in MHB, cells grow faster in this medium. The doubling time was, as expected from the growth rate results, higher for LTB ($1.75\pm0.79$ h) than it was for MHB ($1.26\pm0.16$ h) ($P > 0.05$). Koukkidis et al. (2016) studied the influence in *Salmonella* growth using salad leaf juices. The authors obtained higher O.D. values when using spinach juice, demonstrating that this fluid promoted cell growth, as well as cell adhesion to the salad leaves. The higher O.D. obtained for the LTB is in agreement with these results of Koukkidis et al. (2016).

The higher O.D. can be also a result of other components produced by *E. coli* cells. The cells can be producing EPS that affect the absorbance, consequently affecting the results.

SEM inspections were performed to assess differences in the cell morphology due to the distinct growth media. Figure 4.18 shows that *E. coli* grown in MHB are longer and thinner (2.37±0.58 µm × 0.48±0.13 µm), while cells grown in LTB are shorter and larger (1.67±0.33 µm × 0.57±0.08 µm). Additionally, there are more total cells in LTB than in MHB, which can help to explain the differences observed in the O.D. measured.

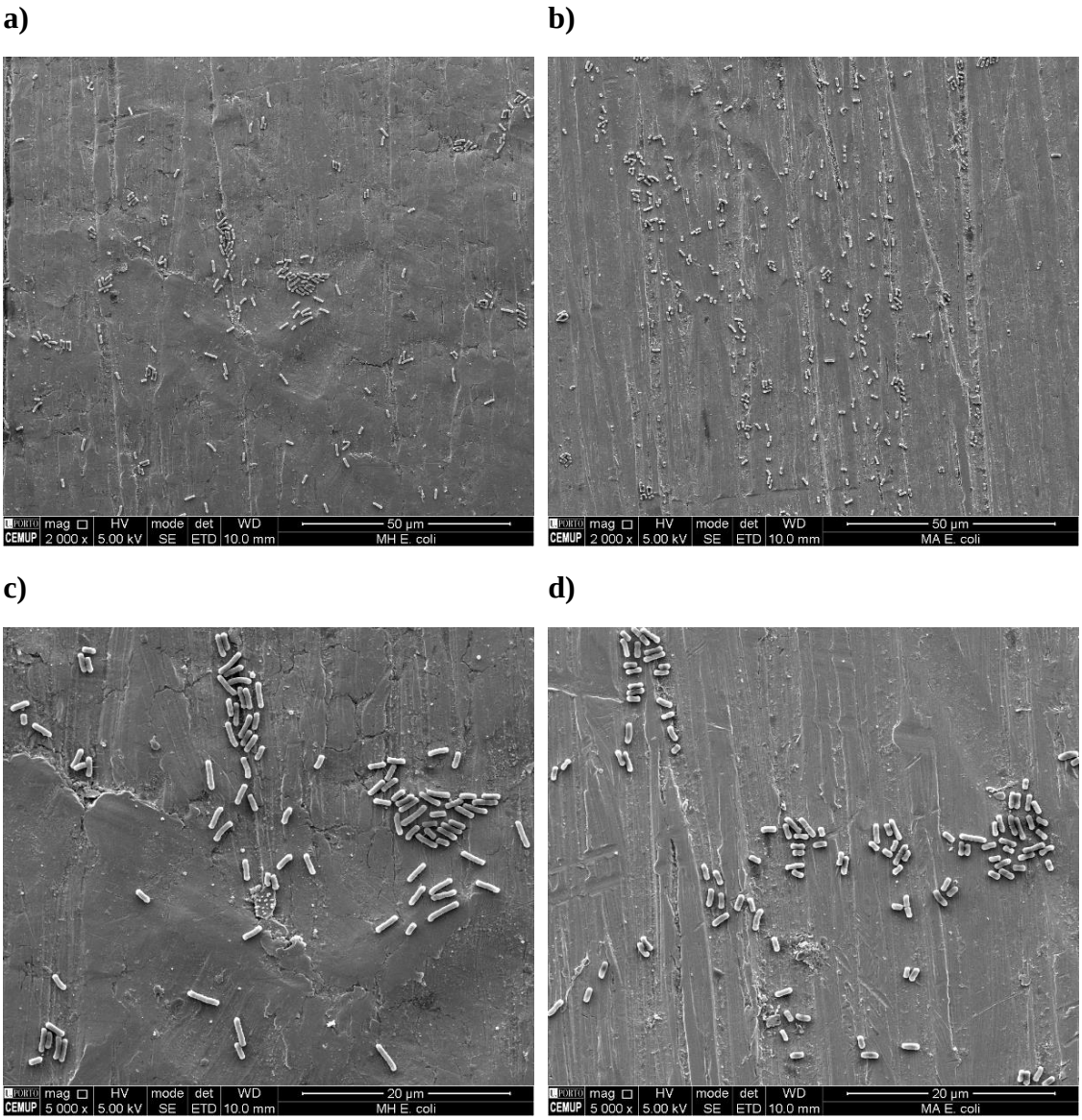


**Figure 4.18.** Scanning electron micrographs of *E. coli* CECT 434 on SS surfaces grown on MHB (a and c) and LTB (b and d). Magnification: 2000× (a and b) (bars = 50 $\mu$m) and 5000× (c and d) (bars = 20 µm).

### 4.5.2. MIC and MBC comparison

The MIC values obtained for SH were 447 and 596 ppm for MHB and LTB, respectively (Table 4.14). When LTB was used, cells became more tolerant to SH and the concentration needed to inhibit microbial growth was higher ($P < 0.05$). The same tendency was observed for the MIC of PA: when LTB was used the concentration necessary to inhibit *E. coli* was higher. Generally, when LTB was used the MIC values obtained were much higher ($P < 0.05$): 596 and 1369 ppm, for SH and PA, respectively. The PA concentration necessary to inhibit cellular growth in LTB was 3 × higher than the concentration needed in MHB. Regarding MBC determination, for SH, the value obtained in MHB was the same as the MIC, however, for LTB the concentration needed to effectively kill the cells was higher ($P > 0.05$) than the MIC. For PA the MBC was always superior to the MIC in both media. Once again, when PA was used the concentration to eliminate the cells was 3 × higher in LTB than the concentration used in MHB. Apparently, using LTB increased *E. coli* tolerance to both disinfectants ($P < 0.05$). Possibly, the components present in LTB, in particular soy peptone and sodium bicarbonate, can be interacting with the biocides, which can justify the distinct results observed.

### 4.5.3. Biofilm formation

Biofilm formation is also an important factor to test, due to its significance in food products and processes (Liu et al., 2013). Bacteria are more resistant to disinfectants when they are in biofilms (Hood and Zottola, 1995, Lewis, 2001). For this reason, biofilm formation in both media was tested and compared. Biofilm formation was characterized with CV staining for mass quantification and with CFU for counting of culturable bacteria. In what concerns CV staining (Table 4.15), the values obtained for both media

**Table 4.14.** MIC and MBC obtained for *E. coli* CECT 434 grown on MHB and LTB

| Medium | Disinfectant | MIC (ppm) | MBC (ppm) |
|---|---|---|---|
| MHB | Sodium hypochlorite | 447 ± 74 [a] | 447 ± 74 [a] |
| MHB | Peracetic acid | 456 ± 0 [a] | 532 ± 91 [a] |
| LTB | Sodium hypochlorite | 596 ± 74 [b] | 670 ± 149 [b] |
| LTB | Peracetic acid | 1369 ± 0 [c] | 1437 ± 221 [c] |

Values are presented as the mean (ppm) ± standard deviation for at least three independent experiments. Different letters within the same column represent statistically different values ($P < 0.05$).

**Table 4.15.** Biofilm formation for *E. coli* CECT 434 in MHB and LTB, evaluated by CV staining

| Medium | O.D. 570 nm | Classification |
|---|---|---|
| MHB | 0.131 ± 0.024 [a] | + |
| LTB | 0.187 ± 0.033 [a] | + |

Values are presented as the mean ± standard deviation for at least three independent experiments. Different letters represent statistically different values ($P < 0.05$). + weak biofilm producers

are similar ($P > 0.05$). It was found that LTB allowed a higher cell adhesion which was followed by a higher biofilm formation ($P > 0.05$), when compared to MHB. To quantify these results the CFU were also accounted (Figure 4.19). None of the results were statistically different from each other: 6.18 log CFU.cm$^{-2}$ in LTB *vs* 6.15 log CFU.cm$^{-2}$ in MHB.

Moreover, *E. coli* CECT 434 demonstrated to be tolerant to both disinfectants in the concentrations tested and in both media. Li et al. (2001) had already verified that *E. coli* was resistant to SH 20 ppm, since they did not achieve considerable log reductions with this disinfectant. It should be noted that the reductions achieved with SH and PA were lower in LTB than in MHB: 0.448 log CFU.cm$^{-2}$ *vs* 0.706 log CFU.cm$^{-2}$ for SH and 0.000 log CFU.cm$^{-2}$ *vs* 0.123 log CFU.cm$^{-2}$ for PA. These results propose that *E. coli* CECT 434 can become more tolerant in LTB. Additionally, for the concentration tested, PA demonstrated to be less efficient in CFU reduction compared to SH ($P > 0.05$). It

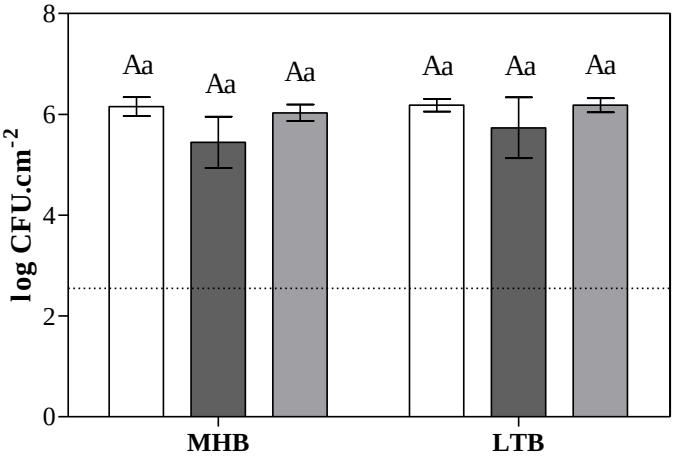


**Figure 4.19.** Log CFU.cm$^{-2}$ achieved after the application of SH and PA at 50 ppm for *E. coli* CECT 434 grown on MHB and LTB. The line indicates the method detection limit (2.55 log CFU. cm$^{-2}$). Different letters represent statistically different values within the same media (A-C) and between both media (a-c) ($P < 0.05$). The error bars represent the standard deviation. MHB - Mueller–Hinton broth; LTB – lettuce broth; □ Control; ■ SH 50 ppm; ■ PA 50 ppm.

should be highlighted that when LTB was used PA 50 ppm had no effect in *E. coli* CFU reduction. Once again, the LTB medium components can be interacting with the disinfectants affecting the results. The results obtained clearly demonstrate that applying a medium similar to lettuce, in chemical composition, will lead to different results than when using MHB. Probably, LTB components are reacting with the biocides hindering the antimicrobial effects. This implies that when disinfectants are tested to define the optimal concentration to eliminate the present microflora, the growth media usually used are not appropriate. In fact, the media typically used do not mimic the actual environment (food surface or bulk medium) in the MPV industry.

# 5

## CONCLUSIONS AND FUTURE WORK

This chapter includes the general conclusions of the thesis and presents the future research.

## 5.1. Conclusions

Foodborne illness outbreaks linked to fresh produce are becoming more frequent and widespread. The types and properties of the chemical agents used for washing, cleaning and disinfection procedures, particularly their toxicity are the key indicators of environmental performance of a MPV industry. In this study, several chemical disinfectants were evaluated (CD, PA, HP, CS, VN and SB) alone and combined with SH on the control of *E. coli* planktonic and sessile cells. The most effective disinfectants tested in planktonic cells were PA (460 ppm) and CD (200 ppm) and the best combination with SH (225 ppm) was obtained with PA (150 ppm). In sessile cells, HP and VN had antagonistic effects in combination with SH whereas SB efficiency was enhanced when combined with SH. Taking into account that SH is considered a major risk for the formation of carcinogenic and mutagenic products (European Comission, 2007), the overall results demonstrate that the reduction of SH concentration in disinfection is possible using alternative biocide combinations. A promising combination would be SH with PA and SH with SB.

Additionally, four chlorine-based disinfectants, including SH, were compared to each other in both planktonic and sessile tests with *E. coli*. SH was the fastest biocide, NaDCC exhibited the higher antimicrobial rates, CD and NaDCC had a similar antimicrobial activity and NEOW had the highest antimicrobial action in planktonic tests. In the biofilm tests NEOW and CD were the biocides that allowed higher log CFU reduction (3.26 and 3.20 log $CFU.cm^{-2}$ for NEOW and CD, respectively). ROS determination proposed that antimicrobial actions of NEOW and SH were related to ROS formation. Therefore, NEOW demonstrated to be more effective than SH in the control of both planktonic and sessile bacteria. Furthermore, in the food industry, the fresh produce is typically disinfected at temperatures around 5 ºC, thus the stability at this temperature is important. NEOW had a longer decay time for chlorine loss (70 days) and the smallest chlorine loss rate (0.013 $ppm.min^{-1}$) at 5 ºC. Consequently, the overall results propose that SH can be replaced by a greener chlorine-based alternative (NEOW) that is more effective in microbial growth control and has lower over time chlorine decay.

Moreover, this study also aimed to bring further understanding on the critical aspects of the microflora present along a MPV process chain, allowing the identification of potentially critical microorganisms. From the MPV plant studied, 47 bacteria were isolated, *Pseudomonas* spp. were predominant and represented 49% of the total contaminants, and 20 different species were identified. Among all the isolates, it was possible to observe that *Pseudomonas* spp. and *R. aquatilis* were present along the process chain and were present in the final product. Moreover, the conveyor belt, at the end of the process, and the air cooling system were found to be a source of contamination requiring attention. Most of the microbial isolates were capable of producing virulence and food spoilage-related molecules (siderophores, gelatinases and proteases). The Gram-negative bacteria were more resistant to the antibiotics (tetracycline, erythromycin and ampicillin) than the Gram-positive ones. *E. bacterium*, *P. oryzihabitans*, *P. putida* and *R. aquatilis* were resistant to two of the antibiotics tested and *S. maltophilia* was resistant to three of the antibiotics tested (tetracycline, erythromycin and ampicillin). *R. erythropolis* was the only one able to aggregate and no evidence of coaggregation was found. Additionally, none of the isolates was able to survive to the simulated gastric environment.

All the bacteria identified are biofilm producers and *Pseudomonas* was the genera that produced more biofilm. Therefore, the results propose that *Pseudomonas* spp. are the resilient microorganisms to target by the decontamination procedures. No foodborne pathogen was isolated, however, some of the isolated bacteria are emerging pathogens (*P. oryzihabitans*, *S. maltophilia* and *R. aquatilis*) and spoilage agents of vegetables (*X. campestris*, *Pseudomonas* spp. and *Erwinia* spp.). When comparing the raw material with the final product, it can be assumed that the current decontamination procedures on site were not completely efficient. The environmental conditions were not adequate for the procedure as the final product had the same microorganism from the beginning (*Pseudomonas* sp.) and additional were found along the process (*R. aquatilis* and *P. poae*). The production plant would certainly benefit from changes in the equipment and processing conditions, particularly in the air cooling system (showing corrosion) and conveyor belts (with obvious visual contamination during processing and a rough polymeric material certainly facilitating bacterial adhesion), and the replacement of SH by alternative decontaminants. However, when using SH and PA at 50 ppm it was proved that these disinfectants were not suitable for biofilm removal or elimination. This can be detrimental in the cases where the suitable conditions are provided and the bacteria are

able to recover and regrow. Which was observed after disinfectant exposure, proposing the modest action of the selected disinfectants in biofilm control and the need for alternative and improved strategies for disinfection of RTE vegetables industry.
Furthermore, the microorganisms used to test disinfectant strategies are typically reference strains. However, food isolates behave different than reference strains. In order to understand the magnitude of such differences, a food isolate (*P. oryzihabitans*) was studied and compared with *E. coli* CECT 434, the reference strain selected. It was possible to assess that *P. oryzihabitans* was more resistant to SH (higher MIC and MBC). Antibiotic resistance was also higher for *P. oryzihabitans*. In what concerns biofilm formation and elimination both bacteria presented similar results, however, *P. oryzihabitans* was less susceptible to PA. This can be an additional indicative that isolates should be tested for alternative disinfectants, since this chemical seemed a promising alternative and biofilm elimination was lower. Furthermore, the food isolate produced much more siderophores which are a virulence indication. Consequently, it can be stated that the reference strains do not reflect the behavior of these microorganisms found in the industrial scenario.

To try to mimic the MPV industry a new artificial medium (LTB) that mimics the lettuce chemical composition was tested and compared to a commonly used medium (MHB) in antimicrobial tests. *E. coli* CECT 434 formed more biofilm in LTB than in MHB and the log CFU reductions achieved with SH and PA were lower in LTB than in MHB. When LTB was used the cells demonstrated higher tolerance to SH and PA. For PA the concentration necessary to inhibit cellular growth was 3 × higher. The results obtained clearly demonstrate that applying a medium similar to lettuce, in chemical composition, for microbiological tests, will lead to different results. This implies that when disinfectants are tested to define the optimal concentration to eliminate the microflora, the media usually used are not appropriate. The media used are not similar and do not mimic the actual environment in the MPV industry.

## 5.2. Future work

The present study provides an insight in MPV contamination, as it was possible to determine the critical points in the food process chain and which were the more critical microorganisms. Also, it allowed the study of several disinfectants in comparison with the in-use SH. Although many important results were obtained with this work, there are

always improvements in terms of new disinfectants and new combinations that can be explored. Examples of alternatives and further exploration are presented below.

It was already stated in this work that physical methods used for disinfection have several advantages, such as environmental friendly, not toxic and safe. Additionally, it was described that these methods should not be applied alone but in combination with other methods, including chemical-based methods. Thus, to explore these safe methods, it is proposed that UV-C light should be combined with disinfectants, particular NEOW. This chemical agent was effective in microbial growth control and was stable over time considering chlorine decay. Another physical-based method that should be tested in combination with NEOW is the application of US. The use of US would promote cell detachment from the surfaces and with the free radicals formation combined with NEOW the disinfection action would be increased and effective. The regrowth phenomena would possibly be reduced.

Furthermore, the search for alternative chemicals is still current. Therefore, there are other chemical agents that can be tested, such as bismuth thiols. These chemicals are highly effective and have a low toxicity. They have been used for the treatment of gastritis, colitis and syphilis (Yang and Sun, 2007). As examples there are bismuth-1,2-ethanedithiol (BisEDT), 2,3-dimercapto-1-propanol (BisBAL) and bismuth-2-mercaptoethanol (BisME). It has been demonstrated that they have antibacterial action against biofilms of *P. aeruginosa* and *S. aureus* (Halwani et al., 2009, Folsom et al., 2011, Varposhti et al., 2014). It would be interesting to study their action against the MPV isolates and assess their food safety aspects and their sustainable use at industrial scale.

The spoilage can be caused mostly by the microflora present in the MPV (Gram et al., 2002). Therefore, it would be interesting to extend the knowledge on the spoilage potential of the isolated bacteria, since the tests made were to attest the pathogenicity. In fact, although the health issue is of upmost importance, the spoilage potential is also critical. Spoiled produce has severe economic consequences. The browning in the vegetables leaves is caused by the microorganisms that can produce proteases, lipases, or lecithinases, that affect the physical and chemical properties of the produce, causing changes in the flavor or odor (Dogan and Boor, 2003). It has been described in the literature (Degl'Innocenti et al., 2005, Barth et al., 2009) that the enzyme polyphenol oxidase (PPO) is the main responsible for tissue browning. This enzyme catalyzes the phenols oxidation to quinones that can spontaneously polymerize to brown pigments

(Degl'Innocenti et al., 2005). The purification of PPO from fruits and vegetables has been studied (Yang et al., 2000, Rocha and Morais, 2001, Mahmood et al., 2009, Mukherjee et al., 2012, Shanti et al., 2014), but not the extraction directly from the bacteria. Some of the isolated bacteria, *Pseudomonas* sp., *Erwinia* sp., *R. aquatilis* and *X. campestris*, have been described as spoilage (Liao, 1989, Gram et al., 2002, Barth et al., 2009, Kalaskar et al., 2014). Therefore, it would be of scientific interest to verify if these bacteria are spoilage due to PPO. This could be easily achieved through the enzyme detection by gel electrophoresis (Yang et al., 2000), or spectrophotometric methods (Shanti et al., 2014) or even using matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) (Zamorano et al., 2009).

Additionally, the last practical works performed should be further explored. Regarding the comparison of *E. coli* CECT 434 behavior with the food isolates, other bacteria could be tested, such as *P. putida* since it was able to produce all the virulence factors, or even *R. aquatilis* or *S. maltophilia* that are potentially pathogenic. The work performed with LTB should also be completed. Food isolates could be tested for the same conditions used with *E. coli* CECT 434. Furthermore, actual lettuce extracts could be used for comparison with the medium developed.

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21CFR173.300 (2014). Secondary direct food additive for human consumption. Food and Drug Administration. 173.300 Chlorine dioxides.

21CFR173.368 (2014). Secondary direct food additives permitted in food for human consumption. Food and Drug Administration. 173.368 Ozone.

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