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NATURAL APPROACHES TO CONTROL *Listeria monocytogenes* IN FRESH VEGETABLES

Thesis submitted to *Universidade Católica Portuguesa* to attain the degree of PhD. in Biotechnology, with specialisation in Microbiology

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by

Bárbara Filipa Santos Ramos

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Paula Cristina Maia Teixeira, Assistant Professor

June 2016

Ao Rui,

Por ser tudo para mim e fazer tudo por mim.

Resumo

Existe uma preocupação crescente acerca da segurança microbiológica dos vegetais. A microbiota dos vegetais é normalmente constituída por bactérias não patogénicas para o Homem, que podem estar presentes aquando a sua ingestão. Contudo, os vegetais também podem conter bactérias patogénicas. *Listeria monocytogenes* é uma grande preocupação para a indústria alimentar e para os consumidores, devido à sua ubiquidade e tolerância a diversos fatores de stresse, bem como à sua presença tanto em matérias-primas como em alimentos processados. Os consumidores estão a evitar alimentos com conservantes artificiais, assim é necessário estudar e implementar medidas naturais para diminuir e prevenir o risco de contaminação dos alimentos.

Esta dissertação visa contribuir para gerar dados sobre metodologias alternativas e naturais para reduzir a contaminação de vegetais com *L. monocytogenes*. Os objetivos principais deste estudo são: determinar estratégias naturais para controlar *L. monocytogenes* em vegetais, em ambientes domésticos e de retalho; e desenvolver um sistema de biocontrolo para controlar e inibir *L. monocytogenes* em vegetais.

Determinou-se a atividade antibacteriana, na etapa de lavagem, de várias soluções de vinagre, sobre *L. monocytogenes* inoculada em alface. As soluções de vinagre balsâmico resultaram na maior redução do patogénico, que é similar ou superior às obtidas noutros estudos realizados com alface, usando desinfetantes à base de cloro. Esta abordagem é uma alternativa promissora para reduzir outros patogénicos de origem alimentar presentes em hortícolas, no ambiente doméstico ou no retalho.

Da alface *Iceberg* isolaram-se e estudaram-se bactérias de ácido lático com atividade antibacteriana contra patogénicos humanos. O isolado DT016 exibiu atividade sobre *L. monocytogenes*, *Listeria innocua* e *Enterococcus faecalis.* A bactéria DT016, identificada como *Pediococcus pentosaceus* DT016, produz uma proteína resistente ao calor com um peso molecular de 11 a 17 kDa, semelhante à pediocina AcH (pediocina DT016). A bacteriocina é estável numa vasta gama de valores de pH e mantém a sua atividade contra *Listeria* em condições de refrigeração (4 ºC). Contudo, a atividade antibacteriana foi afetada por algumas enzimas e detergentes (proteinase K, pronase, papaína, pepsina,

tripsina, Triton X-114 e Triton X-100), assim como por temperaturas iguais ou superiores a 80 °C.

A sobrevivência de um cocktail de *L. monocytogenes* presente em alface, rúcula, salsa e espinafre frescos foi estudada durante armazenamento refrigerado. O potencial de *P. pentosaceus* DT016, como uma cultura protetora para inibir *L. monocytogenes* em vegetais frescos, também foi avaliado. Durante o armazenamento refrigerado dos vegetais, o patogénico foi capaz de crescer e registou-se um aumento da sua carga microbiana. Nos vegetais com a cultura protetora, os níveis do patogénico diminuíram durante o armazenamento, e no último dia de armazenamento registou-se uma redução na carga do patogénico de pelo menos 1,4 log UFC/g. Uma abordagem final foi realizada para avaliar o potencial da pediocina DT016 contra *L. monocytogenes* em alface, rúcula, salsa e espinafre frescos. O agente de biocontrolo foi aplicado durante a etapa de lavagem. Após a contaminação dos vegetais frescos com um cocktail de *L. monocytogenes*, a carga microbiana do patogénico foi estudada durante o armazenamento a 4 °C, após lavagem: com água, com uma solução comercial de hipoclorito de sódio (AMUKINA), e com a solução de pediocina. Observou-se que a solução de pediocina reduziu a carga inicial e inibiu a proliferação de *L. monocytogenes*. Em contraste, a bactéria patogénica foi capaz de crescer durante o armazenamento nos vegetais lavados com água e AMUKINA. No final do armazenamento, a carga do patogénico presente nos vegetais lavados com a pediocina foi inferior à dos vegetais lavados com água e AMUKINA, por um valor mínimo de 3,2 e 2,7 log UFC/g, respetivamente.

No geral, esta tese pode possibilitar a compreensão e conceção de abordagens alternativas efetivas para manter a qualidade microbiológica de vegetais, e apresenta alternativas com eficácia maior ou igual à desinfeção à base de cloro na remoção de *L. monocytogenes* nas hortícolas: uma abordagem acessível em ambientes domésticos e de retalho; e abordagens de biocontrolo para controlar a proliferação de *L. monocytogenes* em vegetais frescos.

Palavras-chave: *Listeria monocytogenes*, vegetais, atividade sobre *Listeria*, vinagre balsâmico de Modena, etapa de lavagem, biocontrolo, cultura protetora, bacteriocina, *Pediococcus pentosaceus* DT016, pediocina DT016.

Abstract

There is a growing concern about the microbiological safety of fresh vegetables. Vegetables' natural microbiota is usually non-pathogenic for humans and may be present at the time of consumption. However, vegetables may also harbour human pathogenic bacteria. *Listeria monocytogenes* is a major concern for food producers and consumers, due to its ubiquity and tolerance to diverse stressing factors, as well as its presence in both raw and processed foods. Consumers' are increasingly avoiding consumption of foods treated with artificial preservatives and so natural measures and interventions should be investigated and implemented to prevent and minimize the risk of food contamination.

This dissertation aims at contributing to generate data on alternative and natural approaches to reduce *L. monocytogenes* from vegetables. The main purposes of this research were to ascertain natural strategies to control *L. monocytogenes* from vegetables in the home and retail environment, and to develop a biopreservation system to control and inhibit *L. monocytogenes* in vegetables.

The antibacterial activity of various vinegar solutions, in a washing step, against *L. monocytogenes* inoculated onto lettuce was determined. Balsamic vinegar solutions demonstrated the maximum pathogen reduction capacity; the reductions observed were similar or higher than those achieved with chlorine-based sanitizers evaluated in other studies with lettuce. This is a promising approach for reducing foodborne pathogens present in produce, at home and retail environments.

From Iceberg lettuce, lactic acid bacteria were isolated and screened for antibacterial activity towards human pathogens. The isolate DT016 exhibited activity against *L. monocytogenes*, *Listeria innocua* and *Enterococcus faecalis.* DT016 strain identified as *Pediococcus pentosaceus* DT016, produces a heat-resistant 11 to 17 kDa protein similar to pediocin AcH (pediocin DT016). The bacteriocin was stable in a wide range of pH values and maintained the antilisterial activity at refrigeration temperature (4 ºC). However, its antibacterial activity was affected by some enzymes and detergents (proteinase K, pronase, papain, pepsin, trypsin, Triton X-114 and Triton X-100), as well as by temperatures equal or above 80 ºC.

The fate of a *L. monocytogenes* cocktail in fresh lettuce, rocket salad, parsley and spinach was evaluated during refrigerated storage. The potential of *P. pentosaceus* DT016 as a protective culture to inhibit *L. monocytogenes* was also assessed in the fresh vegetables. The pathogen was able to grow and an increasing load was observed in the vegetables during refrigerated storage. In the presence of the protective culture, the pathogen levels decreased throughout storage in all the vegetables, and at the last day of storage a minimum pathogen reduction of 1.4 log CFU/g was attained.

An ultimate approach was conducted to evaluate the potential of bacteriocin DT016 against *L. monocytogenes* in fresh lettuce, rocket salad, parsley and spinach. The biopreservation agent was applied in a washing step. After contamination of the fresh vegetables with a *L. monocytogenes* cocktail, the pathogen load was studied during storage at 4 ºC, after washing with: water, a commercial sodium hypochlorite solution (AMUKINA) and the bacteriocin solution. It was observed that the pediocin solution reduced the initial *L. monocytogenes* load and inhibited the pathogen proliferation. In contrast, the pathogen was able to grow along storage in the vegetables washed with water and AMUKINA. At the end of storage, the pathogen load in the vegetables washed with the pediocin was lower than in the vegetables washed with water and AMUKINA, by at least 3.2 and 2.7 log CFU/g, respectively.

Overall, this thesis may contribute to a better understanding and development of alternative approaches to maintain the microbial safety of vegetables, and displays alternatives to chlorine disinfection with the similar or higher effectiveness to reduce *L. monocytogenes* present in produce: an accessible approach to be applied at home and retail environments; and biopreservation approaches to control the proliferation of *L. monocytogenes* in fresh vegetables.

Keywords: *Listeria monocytogenes*, vegetables, antilisterial activity, balsamic vinegar from Modena, washing step, biopreservation, protective culture, bacteriocin, *Pediococcus pentosaceus* DT016, pediocin DT016.

Acknowledgments

I would like to express my gratitude to Escola Superior de Biotecnologia of the Catholic University of Portugal, for accepting me as Ph.D. student and for providing the necessary conditions to develop my work.

My word of appreciation goes to my supervisor Dr. Cristina L.M. Silva, for accepting me as her Ph.D. student. She supported and encouraged me during this journey, accompanying me all the way. I am very thankful for the opportunity she gave me to enter in such a nice and multidisciplinary investigation group.

I also would like to thank my co-supervisor, Dr Paula Teixeira, for her helpful and interest in me and in my work. I am very grateful for her guidance, invaluable discussions and specially her confidence in me.

A special word to my co-supervisor Dr. Teresa Brandão. Thank you for the knowledge shared, and especially for the friendship, support, encouragement and concern in all moments. You had always a kind word for me and made me feel appreciated and accomplished.

I gratefully acknowledge Fundação para a Ciência e a Tecnologia for financial support through grant SFRH/BD/42169/2007.

Thanks to Fátima Miller for being such a good friend, sometimes her presence was the only happy moment in a bad working day. I will always miss the good old days and nights spent in the laboratory. I hope that we will have the opportunity to work so closest as before, and have a lot of happy days.

A special word to Ana Carvalheira for being such a close friend, for listening my fears and doubts, and for all the sheering talks in the good and bad moments. I will treasure that moments forever.

To all my friends and colleagues, Joana Fundo, Elisabete Alexandre, Inês Ramos, Sara Oliveira, Sandra Borges, Sara Silva, Joana Barbosa, Rui Magalhães and Vânia Ferreira who shared the laboratory and/or the office with me. Thank you for providing a pleasant working place.

To my colleagues, Helena Albano, Ivone Moreira, Raquel Mesquita and Susana Vidigal, I am grateful for all the helpful insights in my work.

Thanks to all staff of Escola Superior de Biotecnologia, especially Ana Martins and Ricardo Amadeu, who in one way or another contributed to the development of my work.

To all my friends and family a big Thank you for the friendship and support.

To my closest and dearest friend Patrícia Almeida, thank you for being in my life, you are so special to me that I cannot put in words – you are My Person!

I also have to mention Elisângela Costa, I miss so much our talks and our voyages.

To Inês Silva, I am thankful for your smile and happiness. I LOVE when we meet and you always come running to give me a hug. I hope that you never stop doing that. Only you can turn a cloudy into a shining day. I am so grateful for your love and for being your best friend. I love you so much. I´m looking forward to see you grow and maybe become a scientist like me. No matter what, you will always have a big place in my heart.

To my nephew Pedro Santiago, thanks for existing in my life, you always made me feel loved and my love for you is ETERNAL and UNCONDITIONAL.

My deepest gratitude goes to my parents. Even they don't understand this "Scientific World", they felt always proud of me and encouraged me to finish this journey. Thank you for all the support.

To my parents in law, thanks for being so caring and positive.

My last words go to my husband. Rui, I am grateful for the amazing support and unconditional love during the years. My life only makes sense with you by my side and with your constant encouragement in all moments. With you, I can spread my wings and fly, always knowing that my home is in your arms. With you, I feel able to conquer the World. With you and because of you I dream big and I challenge myself. I LOVE YOU with all my heart and soul.

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Objectives

Vegetable´s consumption has grown over the last two decades, especially lettuce that in Europe and the USA is eaten on average by a third of the population once a week. *Listeria monocytogenes* is ubiquitous, frequently isolated from a wide variety of foods. Raw vegetables have been identified as a vehicle of transmission of foodborne outbreaks and play an important role in listeriosis epidemiology.

In attempt to increase vegetables shelf life and safety, several technologies are widely used to inactivate/remove the microorganisms responsible for their contamination and deterioration. Currently, commercial operations use wash treatments with antimicrobials as the only step to reduce microbial populations on fresh produce, where chlorine is the most commonly used sanitizer. However, numerous reports indicate that at the permitted levels chlorine has limited antimicrobial efficacy and it has been associated with the production of potentially toxic substances. In addition, chorine washings affect the produce microbiota and it has been suggested that these treatments promote food susceptibility to pathogenic proliferation, as a consequence of the reduction of the microbial competition.

Improper conditions at home, like temperature abuse, poor cleanliness and inappropriate refrigerator management have also been identified as critical factors in foodborne listeriosis. Thus, it is important to develop strategies to control *L. monocytogenes* in home and retail environments.

An appealing natural and effective approach is the use of vinegar solutions to reduce the risk of pathogen transmission by consumption of contaminated produce.

The use of protective cultures of lactic acid bacteria (LAB) and their natural metabolites is a promising alternative to maintain the food safe. Protective cultures of LAB have been developed in the last decades to increase safety and shelf life of minimally processed vegetables. However, the application of bioprotective cultures at industrial level for commercial products is scarce, because satisfactory conditions under laboratory settings are unable to guarantee the success under real processing and distribution conditions.

Owing to the generally recognized as safe (GRAS) status of LAB, and their antilisterial properties, bacteriocins have gained a lot of attention in food biopreservation applications. Therefore, research on bacteriocins from LAB is important for their potential applications in the food industry and public health contribution.

In this context, the main aims of this thesis were:

- To improve the safety of fresh vegetables consumption in home and retail environments, by evaluating the potential of various vinegar solutions to control *L. monocytogenes* from Iceberg lettuce;
- To isolate, select and identify potential antagonist, LAB bacteriogenic strains, towards *L. monocytogenes* from fresh Iceberg lettuce;
- To characterize the LAB strains and the bacteriocins produced, for their technological features and in order to evaluate their potential as biopreservation agents;
- To evaluate the effect of the addition of the selected LAB on *L. monocytogenes*, LAB and mesophilic bacteria populations present in fresh vegetables (Iceberg lettuce, rocket salad, spinach and parsley);
- To evaluate the effect of a bacteriocin washing solution on the safety and shelf life of fresh vegetables (Iceberg lettuce, rocket salad, spinach and parsley), and to compare it with a commercial hypochlorite sodium solution.

The following scheme intends to demonstrate the thesis outline and the contribution of each chapter to the main objectives.

Schematic presentation of the thesis outline

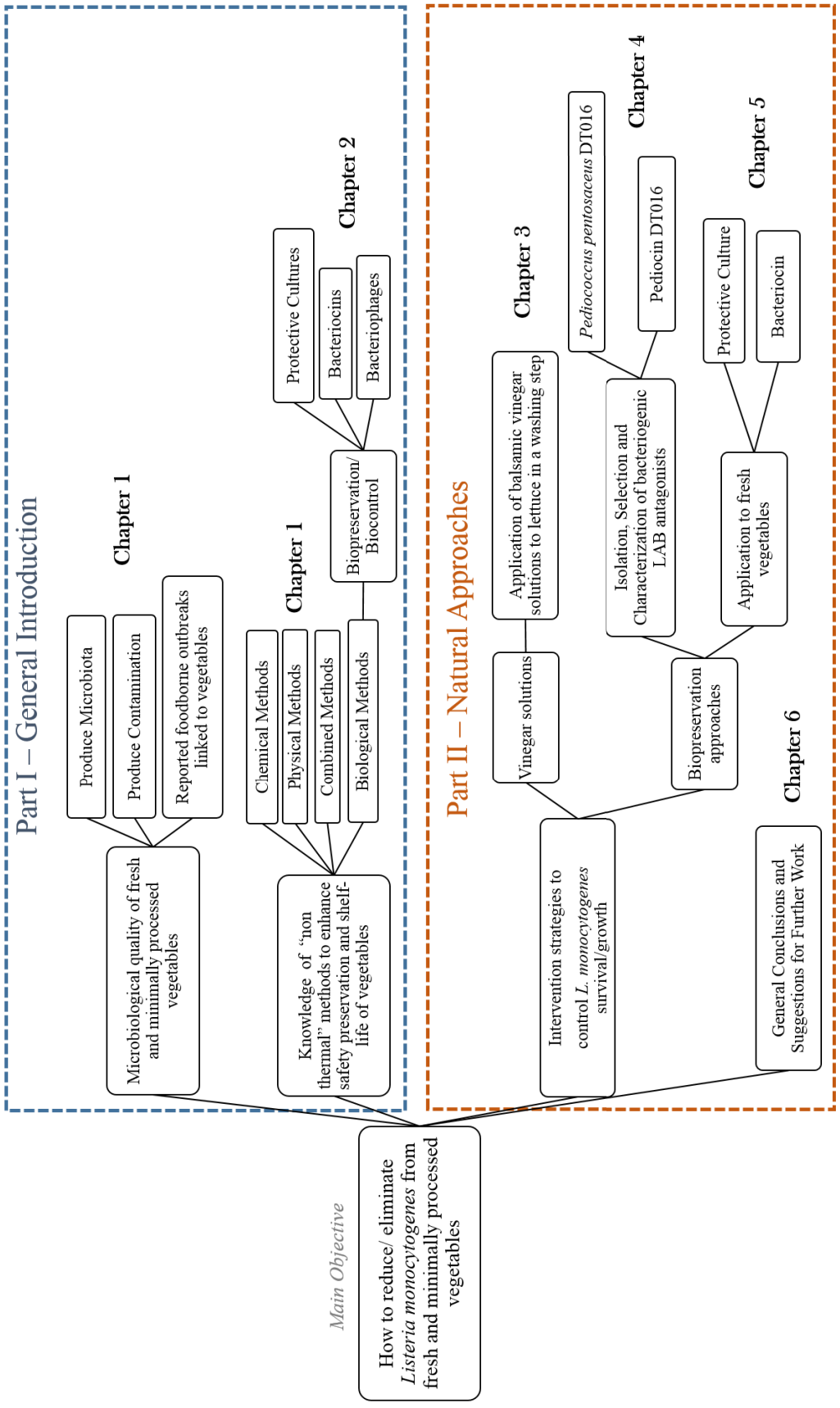

Figure 1. Thesis outline.

Chapter 1

Fresh fruits and vegetables - An overview on applied methodologies to improve its quality and safety

RAMOS, B., MILLER, F. A., BRANDÃO, T. R. S., TEIXEIRA, P. & SILVA, C. L. M.
Innovative Food Science & Emerging Technologies (2013), 20, 1-15.

1.1. Abstract

The consumers demand for fresh fruits and vegetables has increased in recent years. These foods may be consumed raw or minimally processed, and therefore can be a vehicle of several pathogens. The microorganisms most frequently linked to produce-related outbreaks include bacteria (*Salmonella* spp., *Listeria monocytogenes*, *Escherichia coli, and Shigella* spp.), viruses and parasites.

There are many traditional technologies to reduce/eliminate the microorganisms present in food products. However, further research on this topic is still required, since none of the methods reported can control all the parameters necessary to achieve produce with an extending shelf-life, without compromising its quality.

In this paper, an analysis of the alternative and traditional methodologies is made, pointing out the significant advantage and limitations of each technique.

1.2. Industrial Relevance

The significant increase in the incidence of foodborne outbreaks caused by contaminated minimally processed produce in recent years has become of extreme importance. The extensive knowledge of gentle (non-thermal) processes to enhance safety, preservation and shelf-life of these products is crucial for the food industry.

This manuscript presents non-thermal processes that have shown efficient microbial reductions on fresh produce and highlights some of their challenges and limitations.

Keywords: Minimally processed fruits and vegetables; sanitizing methods; quality and safety.

1.3. Introduction

Fruits and vegetables are essential components of the human diet and there is considerable evidence of the health and nutritional benefits associated with their consumption (Abadias et al. (2008), Warriner et al., 2005). Due to the presence of high levels of micronutrients and fibres, their consumption is recommended by many organizations (World Health Organization - WHO, Food and Agriculture Organization - FAO, United States Department of Agriculture – USDA and European Food Safety Authority – EFSA) to reduce the risk of cardiovascular diseases and cancer (Allende et al., 2006a, Ragaert et al., 2004, Su and Arab, 2006, Warriner, 2005).

As a response to consumers' demand for healthy, fresh-like and easy to prepare products, conjoint with consumer lifestyles changes, a wide variety of minimally processed fruits and vegetables (MPFVs) has been developed (Allende et al., 2006a, Froder et al., 2007, Scolari and Vescovo, 2004, Tournas, 2005).

Minimal food processing techniques constitute non-thermal technologies with guarantee of food preservation and safe standards as well as maintaining, as much as possible, the fresh-like characteristics of fruits and vegetables (Allende et al., 2006a, Allende et al., 2006b). Minimally processed products aim to extend the product shelf-life of 5-7 days at 4 ºC, while ensuring food safety and maintaining nutritional and sensory quality (Cliffe-Byrnes and O'Beirne, 2002).

The marketing of these types of foods continues to rise mainly due to their freshness, economic handling and attractive presentation (Little and Gillespie, 2008). They constitute a suitable meal for today's lifestyles, because do not need preparation and provide a great variety of vitamins, minerals and other phytochemicals, which are important in human health (Froder et al., 2007).

Fruits and vegetables require proper handling, preparation and storage in order to take full advantage of their nutrients. When these products are minimally processed, they are submitted to unit operations that include selection, cleaning, washing, trimming, peeling, cutting and shredding, sanitizing and packing. As these operations do not assure the

absence of microorganisms, minimally processed fruits and vegetables, require refrigeration as a primary means of preservation (Froder et al., 2007, Tournas, 2005).
Consumers are becoming more aware about the limitations of commonly sanitizing techniques and are looking for safe food products that suffer minimal processing, with high quality retention. To satisfy these requirements, food industry is currently studying non-thermal techniques such as ozone based treatments, ultraviolet radiation, pulsed light, cold plasma, ultrasounds and novel packaging practices. All these technologies have a great potential in the field of minimally processed foods. However, there is a lack of available information about advantages and limitations of these technologies, when applied to food processing. The efficiency of the processes depends directly on the combination food/contaminant/process and important fresh characteristics to maintain.
This paper gathered information about these novel technologies and the ones commonly used, pointing out their most relevant advantages and limitations. An overview of produce microbiota patterns and outbreaks related to fruits and vegetables is presented, showing the importance of choosing an effective method for microbial inactivation on these products.

1.4. Produce Microbiota

Fresh fruits and vegetables, including plant components as leaves, roots, bulbs and tubers, have different morphology and metabolic functions and consequently provide diverse ecological niches to microorganisms (Brackett, 1999, Burnett and Beuchat, 2000, Ponce et al., 2002). The presence and number of microorganisms differ depending on the type of produce, agronomic practices, geographical area of production, and weather conditions before harvest. Harvest, transportation and further processing and handling of produce can greatly influence the microbiota pattern (Ahvenainen, 1996, Olaimat and Holley, 2012).
The number and type of microorganisms found on fresh produce are highly variable. Mesophilic bacteria are around 10^3-10^9 CFU/g in raw vegetables after harvest, depending

on the produce and the growing conditions (Oliveira et al., 2010, Zagory, 1999). Gram-negative bacteria dominate the microbiota associated with most vegetables, whereas yeasts and moulds are often the majority microbiota of raw fruits (Burnett and Beuchat, 2000, Tournas, 2005). The microbiota of vegetables and fruits is made up largely of *Pseudomonas* spp., *Erwinia herbicola*, *Flavobacterium, Xanthomonas*, and *Enterobacter agglomerans* as well as various moulds, *Alternaria*, *Penicillium*, *Fusarium* and *Aspergillus*. Lactic acid bacteria, such as *Leuconostoc mesenteroides* and *Lactobacillus* spp., are also commonly found. Finally, yeasts as *Torulopsis*, *Saccharomyces* and *Candida* are part of dominant microorganisms, mostly on fruits because of their high sugar content (Caponigro et al., 2010, de Azeredo et al., 2011, Pianetti et al., 2008). *Pseudomonas* spp. normally dominates and may make up 50–90 % of the microbial population on many vegetables (Arvanitoyannis and Stratakos, 2010, Nguyen-the and Carlin, 1994, Zagory, 1999).

1.5. Fruits and Vegetables Contamination

The natural microbiota of raw fruits and vegetables are usually non-pathogenic for humans and may be present at the time of consumption (Ahvenainen, 1996, Food and Drug Administration, 2008). However, during growth, harvest, transportation and further processing and handling, the produce can be contaminated with pathogens from human, animal, or environmental sources (Ahvenainen, 1996, Brandl, 2006, Froder et al., 2007, Sánchez et al., 2012). During peeling, cutting and shredding, the surface of produce is exposed to air and to possible contamination with bacteria, yeasts and moulds. The protective epidermal barrier is breached, which will increase nutrient availability and provide large surface areas that may facilitate microbial growth and consequently decrease the product shelf-life (Conte et al., 2009, Del Nobile et al., 2007, Guerzoni et al., 1996, Muriel-Galet et al., 2013). Additionally, the mechanical damage caused to cells during processing may increase the rate of tissues senescence, reducing their resistance to microbial spoilage (Badosa et al., 2008, Barry-Ryan and O'Beirne, 1998, Garg et al.,

1990). Another concern is the possible formation of foodborne pathogen biofilm´s on plant tissues enabling these to survive in harsh environment and may decrease the efficacy of commonly used sanitizers (Critzer and Doyle, 2010).

As a result, these products can be a vehicle of transmission of bacterial, parasitic and viral pathogens, capable of causing human illness.

The incidence of foodborne outbreaks caused by contaminated fresh fruits and vegetables has increased in recent years. The pathogens most frequently linked to these products contamination and human illness are included in Table 1.

Figure 1 includes collected information of produce outbreaks, which is based on approximately 110 scientific papers and reports by CDC (Centers for Disease Control and Prevention), FDA (Food and Drug Administration) and WHO (World Health Organization).

From about 1100 produce reported outbreaks, in which an etiological agent was identified, 53.0 % were caused by bacteria, 42.5 % by viruses and 4.5 % by parasites.

The produce most associated with outbreaks is the salad, since it has all kind of mixed vegetables. Included in the "other vegetables" group are corn, beans, pepper, soy, radish and onion. In addition, WHO categorized lettuce and salads (all varieties), leafy vegetables (spinach, cabbage, raw watercress) and fresh herbs highest priority in terms of fresh produce safety from a global perspective (FAO/WHO, 2008, Goodburn and Wallace, 2013).

Cantaloupe, cucumber, mamey and pawpaw are fruits included in the "other fruits" group.

As it can be observed in Figure 1, the microorganisms of main concern in produce outbreaks are the Norovirus and *Salmonella* spp. For this reason, several studies had been conducted to investigate the prevalence and contamination level of these microorganisms on fresh produce (Baert et al., 2011, Elviss et al., 2009, Patel et al., 2009, Stals et al., 2012).

Contamination of fresh fruits and vegetables is of special concern, because such produce is likely to be consumed raw, without any type of microbiologically lethal processing, thus posing a potential safety problem (Carrasco et al., 2012, Zweifel and Stephan, 2012). Safe production methods and proper disinfection/decontamination procedures are

therefore critical steps in ensuring the safety of ready-to-eat fresh fruits and vegetables (Artés et al., 2009, Bharathi et al., 2001, Selma et al., 2008b).

Table 1. Major fruits and vegetables pathogens associated with outbreaks (Abadias et al., 2012, FAO/WHO, 2008, Olaimat and Holley, 2012, Senorans et al., 2003, Seow et al., 2012, Van Boxstael et al., 2013, Warriner, 2005, Zhao, 2005)

Pathogen		Product
Bacteria	***Clostridium botulinum***	Cabbage, pepper, garlic, potato and carrots
	***Escherichia coli* O157:H7**	Alfalfa sprouts, cabbage, celery, coriander, watercress, lettuce, cabbage, berries, melons, and apple juice
	Listeria monocytogenes	Bean sprouts, cabbage, chicory, cantaloupe, eggplant, lettuce, potatoes, radish and lettuce
	***Salmonella* spp.**	Alfalfa sprouts, artichokes, beet leaves, celery, cabbage, cantaloupe, cauliflower, eggplant, endive, fennel, green onions, lettuce, mung bean sprouts, mustard cress, pepper, salad greens, spinach, unpasteurized fruit juice, tomatoes, watermelon, mamey and mango
	***Shigella* spp.**	Celery, lettuce, green onions, salad vegetables and parsley
	***Staphylococcus* spp.**	Lettuce, parsley, radish, salad vegetables and seed sprouts
	Vibrio cholerae	Cabbage and coconut milk
	Yersinia enterocolitica	Carrots, cucumbers, lettuce and tomatoes
Viruses	**Norovirus**	Lettuce, green onions, watercress, sliced melon, salads, diced tomatoes and fresh-cut fruit
	Hepatitis A	Lettuce, green onions, watercress, raspberries, frozen strawberries and berries
Protozoa	***Cryptosporidium* spp.**	Lettuce, onions and green onions
	***Cyclospora* spp.**	Lettuce, onions, green onions, raspberries and blackberries

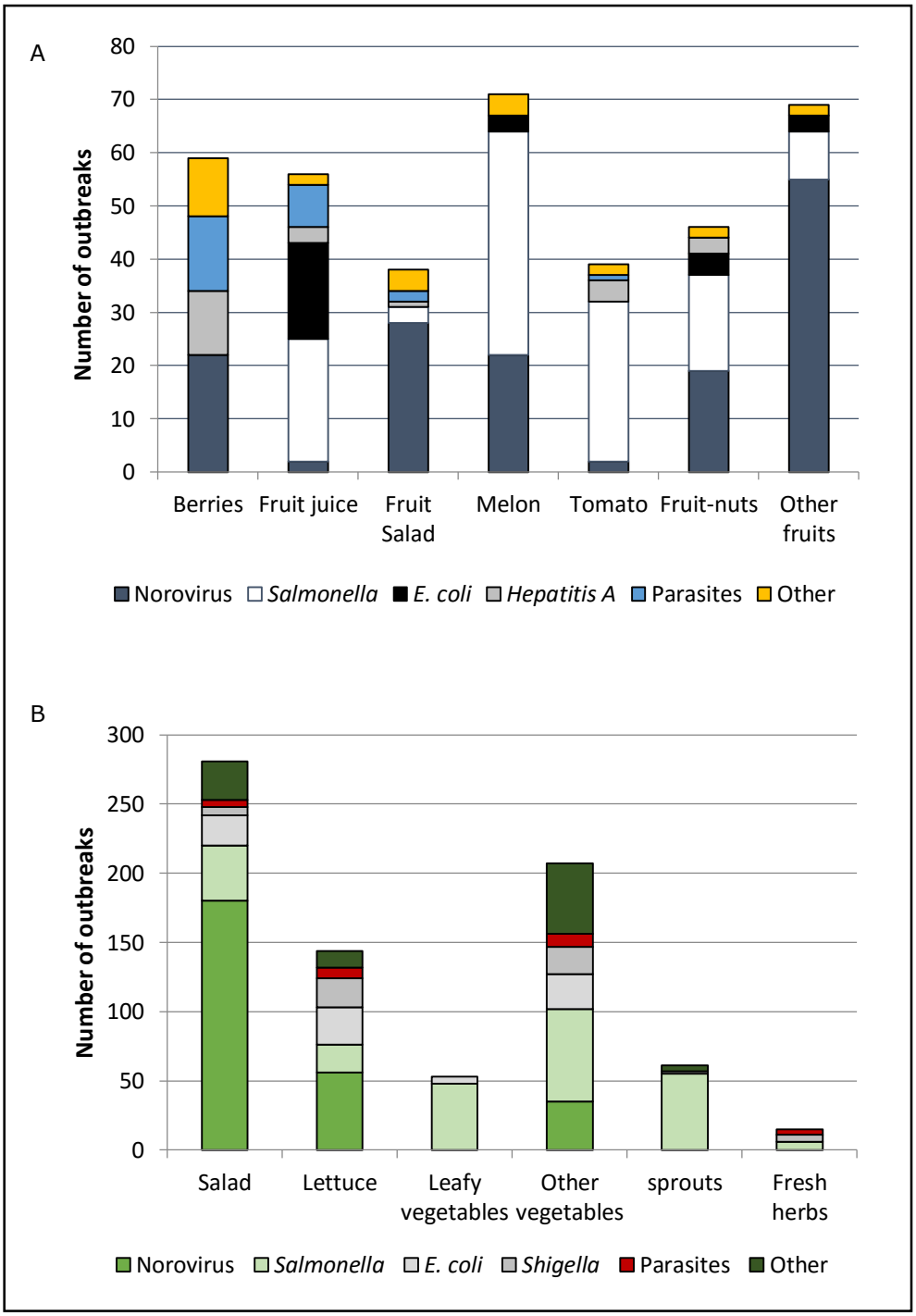

Figure 1. Reported foodborne outbreaks in fruits (A) and vegetables (B).

1.6. Minimally Processed Fruits and Vegetables (MPFVs) Shelf-life

Fruits and vegetables are among the most perishable foods in the market. They are rich in carbohydrates and poor in proteins, with pH values ranging from 7.0 to slightly acidic, and exhibit a characteristic high water activity. These conditions make the produce adequate habitats for several bacteria, yeasts and moulds (Del Nobile et al., 2008, Gómez-López et al., 2005, Selma et al., 2008a). The resulting spoilage of fruits and vegetables is characterized by a brown discoloration, necrosis, loss of texture, exudation and / or production of off-odours or off-flavours (Ponce et al., 2002).

Minimally processed produce, due to processing operations that alter the physical integrity of these products, are more perishable than the original raw materials (Sanz et al., 2002). The raw produce is expected to have a shelf-life of several weeks or months, while MPFVs have only a very short storage life of 4 to 7 days. Their shelf-life depends on various factors such as fruit and vegetable initial quality, production technology and the number and interactions among microbial groups (Watada and Qi, 1999). Although MPFVs should have storage lives up to 21 days, ethylene production, respiratory activity, enzymatic and non-enzymatic browning and nutrient release from cells that are stimulated by produce injuries, make this goal difficult.

The understanding of the processes that result in quality degradation is essential to develop technologies to extend MPFVs shelf-life and to maintain quality during processing and distribution (Pianetti et al., 2008).

1.7. Intervention Methods to Extend Shelf-life and Enhance Safety

There are a variety of methods used to reduce microorganism's population on whole and fresh-cut produce. The best method is to prevent contamination in the first place. However, this is not always possible and the use of techniques that reduce/eliminate pathogens is of extreme importance to prevent foodborne outbreaks (Corbo et al., 2006).

There are a number of chemical (Table 2) and physical (Table 3) methods that were proved to be moderately efficient in reducing the indigenous microbiota and also the contaminating pathogens (Parish et al., 2003). Chemical methods of cleaning and sanitizing produce surfaces usually involve the application of mechanical washing in the presence of sanitizers, followed by rinsing with potable water (Wei et al., 2006). Physical methods are effective at removing bacteria from plant surfaces by the use of shear forces (Allende et al., 2007, Gil et al., 2009).

1.7.1. Chemical methods

Several sanitizing agents may be used for fruits and vegetables washing with the intention of reducing the risk of microbial contamination, helping in the prevention of postharvest diseases and foodborne illness.

This section will describe, in more detail, the chemical methods industrially available for produce decontamination. An overview of the major advantages, limitations and effectiveness for each method is presented in Table 2.

1.7.1.1. Chlorine (Hypochlorite)

In fruits, vegetables and fresh-cut produce, chlorine rinses are frequently used with concentrations varying from 50 to 200 ppm and with typical contact times of less than 5 min (Artés et al., 2005, Rico et al., 2007). Although chlorine is the most commonly used sanitizer, it is inactivated by organic material and during production can also lead to the liberation of chlorine vapours and formation of chlorinated by-products (DBPs), with potential adverse health effects (Parish et al., 2003, Rico et al., 2007). However, the benefits of chlorine use for the produce industry outweigh the concerns of potential formation of harmful by-products. Studies have shown that chlorine rinses can decrease the bacterial load by values ranging from <1 log CFU/g to 3.15 log CFU/g, depending on

inoculation method, chlorine concentration, contact time, and the target bacteria (Baur et al., 2005, Beltrán et al., 2005b, Casteel et al., 2008, Hua and Reckhow, 2007).

1.7.1.2. Chlorine dioxide

As an alternative to chlorine, raw fruits and vegetables can be sanitized with chlorine dioxide (ClO_2) (up to 3 ppm), which has an oxidant capacity 2.5 times greater. In addition, ClO_2 does not participate in chlorination reactions that result in harmful by-products (Keskinen et al., 2009). Although chlorine dioxide reacts directly with cells amino acids and RNA, it is not clear whether it attacks the cell structure or the acids inside the cell. As drinking water disinfectant, it is highly effective against pathogenic organisms such as *Legionella*, *Giardia* cysts, *E. coli*, *Cryptosporidium* and viruses by preventing protein formation (Francis and O'Beirne, 2002, Fu et al., 2007, Gómez-López et al., 2007a, Rico et al., 2007). For raw produce, this method is not so efficient at permitted concentration levels.

ClO_2 gas has received attention due to its greater penetration ability than liquid. Several studies showed the efficacy of this gas on produce surface decontamination, particularly against pathogens like *E. coli* O157:H7 and *L. monocytogenes* (Du et al., 2003, Gómez-López et al., 2008, Han et al., 2000, Mahmoud et al., 2007, Singh et al., 2002). Different factors can influence the lethality of the ClO_2 gas treatment, such as ClO_2 gas concentration, time of exposure, relative humidity, and temperature.

ClO_2 is a promising non-thermal technology for reducing pathogenic and spoilage bacteria on fresh produce. However, research efforts in this area are still required (Mahmoud et al., 2007).

1.7.1.3. Acidified sodium chlorite

This compound can be used on raw fruits and vegetables as a spray or dip in the range of 500 to 1200 ppm. Applications of acidified sodium chlorite showed a substantial antimicrobial effect against *E. coli* O157:H7 and *Salmonella* inoculated onto cantaloupes, honeydew melons and asparagus spears. However, the number of studies is still limited,

and there is a need for more information on the general usefulness of using acidified sodium chlorite in produce sanitizing (Artés et al., 2009, Beuchat, 1998, Parish et al., 2003).

1.7.1.4. Bromine and Iodine

These chemicals showed antimicrobial activity although some health related issues limits their use on produce.

Bromine has synergistic antimicrobial relationship when added to chlorine solutions, but there are safety concerns about the production of brominated organic compounds and their impact on human and environmental safety (Beuchat, 1998, Parish et al., 2003).

Elemental iodine and non-ionic surfactants or carriers combinations-iodophors, have a broad spectrum of antimicrobial activity and are less corrosive than chlorine at low temperatures. However, iodine-containing solutions used as direct contact produce sanitizers are limited due to a reaction between iodine and starch that result in a blue-purple colour (Ayala-Zavala and González-Aguilar, 2010, Parish et al., 2003).

1.7.1.5. Trisodium phosphate (TSP)

Solutions with 15 % TSP are effective in *Salmonella* inactivation on tomato surface. However, even at 15 %, only about a 2 $\log_{10}$ reduction was achieved (Beuchat, 1998, Zhuang and Beuchat, 1996). It was concluded that the use of TSP as a disinfectant for removal of *Salmonella* from the surface of mature green tomatoes has good potential. The use of TSP to remove *L. monocytogenes* from shredded lettuce was demonstrated to be less promising (Parish et al., 2003, Weissinger et al., 2000). Solutions containing more than 10 % TSP damaged the sensory quality of lettuce. It should be noted that the pH of TSP solutions is around 11-12, limiting their commercial application as a disinfectant of fruits and vegetables (Parish et al., 2003).

1.7.1.6. Quaternary ammonium compounds

Quaternary ammonium compounds commonly called "QAC" are cationic surfactants that are able to penetrate food contact surfaces more readily than other sanitizers. Their antimicrobial activity is greater against the fungi and gram-positive bacteria than gram-negative bacteria (Aase et al., 2000, To et al., 2002). Although they are not approved for direct food contact, QAC may have some limited usefulness on whole produce, since the product must be peeled prior to consumption (Parish et al., 2003). As mentioned for iodine compounds, QAC direct food contact would require regulatory approval and a demonstration that the produce is safe for consumption (Dunn, 1949, Rossmoore, 2001).

1.7.1.7. Organic acids

Organic acids (e.g. lactic, citric, acetic or tartaric acid) have been described as strong antimicrobial agents due to environment pH reduction, disturbance of membrane transport and/ or permeability, anion accumulation, or a reduction in internal cellular pH (Parish et al., 2003). Less direct antibacterial activities include interference with nutrient transport, cytoplasm membrane damage resulting in leakage, disruption of outer membrane permeability, and influence on macromolecular synthesis (Beuchat, 1998, Inatsu et al., 2005, Miller et al., 2009). Citric and ascorbic acids are commonly used in fruits and vegetables washing and added in fruit juices (Velazquez et al., 2009).

1.7.1.8. Hydrogen peroxide (H_2O_2)

This compound possesses bacteriostatic and bactericidal activity due to its strong oxidizing power and also due to the generation of cytotoxic agents (hydroxyl radical). It is used as antimicrobial or bleaching agent in the range of 0.04–1.25 % up to 80 ppm in produce wash water (Akbas and Olmez, 2007, Alexandre et al., 2012b, Hwang et al., 2001).

For a better effectiveness of this compound, H_2O_2 concentrations of 2-4% should be used. Lower concentrations (1-2 %) are not efficient in reducing the bacterial load and higher

concentrations (4-5 %) interfere with the overall quality of the produce (Beltrán et al., 2005b, Ölmez and Kretzschmar, 2009, Rico et al., 2007). Although the antimicrobial efficacy of this H_2O_2 can be comparable to 100-200 ppm of chlorine treatment at room temperature, higher microbial reductions were achieved with H_2O_2 and a higher overall quality was maintained when higher temperatures (50-60 ºC) were applied (Parish et al., 2003).

1.7.1.9. Peroxyacetic acid

It is a combination of peracetic acid (CH_3CO_3H) and hydrogen peroxide (H_2O_2), usually commercialized as a liquid. It is a strong oxidant agent and is used to wash fruits and vegetables in concentrations up to 80 ppm. Peroxyacetic acid is effective on the inactivation of pathogenic microorganisms in suspension at lower concentrations than the ones required when using chlorine. However, studies revealed that 80 ppm of peroxyacetic acid in wash water is not sufficient to obtain a substantial reduction in the microbial load of fresh-cut fruits and vegetables (Artés et al., 2009, Rico et al., 2007, Sapers, 2006).

1.7.1.10. Calcium-based solutions

These solutions are widely used for delicate fruits and products with high senescence index, as it maintains produce firmness. One of the compounds most utilized is calcium lactate, because it has antibacterial properties due to its ability to uncouple microbial transport processes. In a study with fresh-cut lettuce and carrots this compound demonstrated the same effectiveness as chlorine in reducing microbial load (Martin-Diana et al., 2005a, Rico et al., 2007).

1.7.1.11. Ozone

Ozone is a strong antimicrobial agent with high reactivity and penetrability. When used in water, ozone concentrations range from 0.03 to 20.0 ppm. When used in the gas form,

the concentration reaches higher doses such as 20,000 ppm (Manganaris et al., 2006, Martin-Diana et al., 2005b, Rico et al., 2007, Saftner et al., 2003).

Ozonated water has been commonly applied for sanitation purposes of fresh-cut vegetables achieving some microbial reductions and extending the produce shelf-life (Alexandre et al., 2011a, Alexandre et al., 2012a, Alexandre et al., 2011b, Miller et al., 2013).

Several studies showed that gaseous ozone is generally more effective than in aqueous solutions (Klockow and Keener, 2010). This treatment was effective against pathogenic and spoilage microorganisms, while assuring an acceptable product quality (Al-Haddad et al., 2005, Baur et al., 2005, Beltrán et al., 2005a, Hua and Reckhow, 2007, Ölmez and Akbas, 2009, Parish et al., 2003, Pascual et al., 2007).

Other studies are dealing with in-package gaseous ozone action. Results indicated that this treatment is very effective against *E. coli* O157:H7, also extending produce shelf life. However, in some products, like spinach, notable colour degradation can occur (Al-Haddad et al., 2005, Klockow and Keener, 2010 , Oner et al., 2011).

1.7.1.12. Electrolysed water (EW)

There are two types of electrolyzed water with sanitizing properties: acidic electrolyzed water or electrolysed oxidising water (AEW) and neutral electrolyzed water (NEW). These solutions are conventionally generated by electrolysis of aqueous sodium chloride (0.5-1.0 % NaCl), and an electrolysed acidic solution (AEW) or an electrolysed basic solution (NEW) is produced at the anode and cathode, respectively.

The AEW has a strong bactericidal effect on pathogenic and spoilage microorganisms (Selma et al., 2008a). This effect is attributed to its low pH (2.1-4.5), high oxidation-reduction potential (higher than 1000 mV), and the presence of active oxidizers such as hypochlorous acid (Keskinen et al., 2009, Rico et al., 2007).

Electrolysed basic solution has also a strong bactericidal effect, with pH values ranging from 5.0-8.5 and oxidation–reduction potential values ranging from 500 to 700 mV (Graça et al., 2011).

Table 2. Chemical methods used for the preservation of minimally processed vegetables and fruits

Method	Advantages	Limitations	Effectiveness	References
Chlorine (Hypochlorite)	- Low cost - Easily available - Long history of use	- Liberation of chlorine vapours during production and formation of chlorinated by-products (DBPs) with potential adverse health effects - Efficacy is affected by the presence of organic matter - Corrosive - Activity pH dependant - Sensitive to temperature, light and air - Banned in some European countries	- Very high concentrations may not eliminate all the pathogens on produce - Chlorine dosages (50-200 ppm) and contact times (1-2 min) used result in 1 to 2 $\log_{10}$ bacterial inactivation - Some resistance by bacterial spores and protozoan oocysts	(Baert et al., 2011, Brackett, 1999, Burnett and Beuchat, 2000, Butot et al., 2007, De Roever, 1998, Harris et al., 2003)
Chlorine dioxide	Aqueous - Higher antimicrobial efficacy at neutral pH than chlorine - Effectiveness less pH dependant compared to chlorine - Fewer potentially hazardous DBP formation than chlorine - Less corrosive than chlorine and ozone - Can delay ripening of produce due to ethylene elimination and inhibiting its production - Aqueous ClO_2 treatments (4, 6 and 8 mg/l) exhibited anti-browning effects in apple juices	Aqueous - Not efficient at permitted levels for fresh produce - Requires on-site generation - Explosive - Not permitted for cut produce in USA and not regulated in EU - Final water rinsing is required after treatment - More iodinated DBP formation than chlorine if iodide exists in water - Formation of specific by-products, chlorite and chlorate - Requires monitoring in indoor applications	Aqueous - Studies revealed that much higher concentrations are needed to have a significant reduction in the microbial load of fruits and vegetables - Reduction of a few logs reported when used at permitted concentrations	(Baur et al., 2005, Beltrán et al., 2005b, Casteel et al., 2008, Sanz et al., 2002, Ukuku, 2006, Vandekinderen et al., 2008, Wei et al., 2007)

Table 2 (continued). Chemical methods used for the preservation of minimally processed vegetables and fruits

Method	Advantages	Limitations	Effectiveness	References
Chlorine dioxide (continued)	ClO_2 gas - Effective in a wide pH range - Great penetration ability	ClO_2 gas - Decomposes into chlorine and oxygen - Must be produced in situ - Activity affected by gas concentration, time of exposure, humidity and temperature	ClO_2 gas - Inactivates *E. coli* O157:H7 and *L. monocytogenes*	
Acidified sodium chlorite	- Greater efficacy than hypochlorite due to low pH	- Little information on production of chlorinated by-products - Limited amount of research conducted	- Inhibited enzymatic browning on fresh-cut produce - Usefulness for produce needs further research	(Allende et al., 2009, He et al., 2008, Luo et al., 2011)
Bromine	- Possible synergy with chlorine compounds	- Information lacking on production of brominated by-products and their potential health effects	- Not widely used as a sanitizer	(Beuchat, 1998, Parish et al., 2003)
Iodine	- Less corrosive than chlorine at low temperature - Broad spectrum - Iodophor less volatile than iodine	- Stains commodities and equipment - Corrosive above 50°C - No direct contact use on produce	- More active against vegetative cells than bacterial spore	(Ayala-Zavala, et al., 2010; Parish, et al., 2003)
Trisodium phosphate	- Less corrosive than most other compounds	Not efficient against *Listeria* - Has very high pH (11-12)	- Concentrations between 1 and 15 % yielded reductions in pathogen populations from 0 to 6 logs - *Listeria* relatively resistant	(Beuchat, 1998; Weissinger, et al., 2000; Zhuang, et al., 1996)

Table 2 (continued). Chemical methods used for the preservation of minimally processed vegetables and fruits

Method	Advantages	Limitations	Effectiveness	References
Quaternary ammonium compounds	- Colourless, odourless - Stable at high temperature - Noncorrosive - Good penetrating ability - Relatively stable to organic compounds	- Limited usefulness at low pH (<6) - Not compatible with soaps or anionic detergents - Costly	- Most effective against fungi and gram-positive bacteria than gram-negative bacteria, except *Salmonella* and *E. coli*	(Parish, et al., 2003; Reynolds, 1991; Rossmoore, 2001)
Organic acids (lactic, citric, acetic, tartaric or ascorbic acid)	- Easy to use - Economical, depending on type of acid and use - No toxicity - Allowed for organic products	- Interferes with the sensory quality - Relatively lower antimicrobial efficacy - Low pH use only - Antimicrobial effect dependent upon type of acid and strain of microorganism - Disinfection with organic acids in fresh-cut industry has an impact on the wastewater quality	- The exposure times needed for a significant reduction in microbial load are very long (5-15 min)	(Benarde et al., 1965, Francis and O'Beirne, 2002, Fu et al., 2007, Singh et al., 2002, Zhong et al., 2006)
Hydrogen peroxide (H_2O_2)	- No harmful DBP formation - No residue production - Not corrosive at permitted levels	- Phytotoxicity against some products like lettuce and berries - Negative impact on overall quality - Low antimicrobial efficacy at permitted levels for vegetables - Less effective against yeasts, fungi and viruses - Not allowed for organic products - Requires the removal of residual H_2O_2 after processing	- At low concentrations (1–2 %), H_2O_2 is not efficient in reducing the pathogenic bacterial counts on the fresh produce - At high concentrations (4–5 %) it interferes with the overall quality of the produce	(Akbas and Olmez, 2007, Alexandre et al., 2012b, Hwang et al., 2001, Ölmez and Kretzschmar, 2009)

Table 2 (continued). Chemical methods used for the preservation of minimally processed vegetables and fruits

Method	Advantages	Limitations	Effectiveness	References
Peroxyacetic acid	- No harmful DBP formation - Efficacy is not affected by water organic load - Efficacy unaffected by temperature changes - Good antimicrobial properties at low temperatures in the pH range (5-8) - Not corrosive at permitted levels	- Low antimicrobial efficacy at permitted levels for vegetables	- At the highest concentration permitted (80 ppm), peroxyacetic acid in wash water is not sufficient to obtain a substantial reduction in the microbial load of fresh-cut fruits and vegetables	(Sapers, 2009, Sapers and Jones, 2006, Vandekinderen et al., 2009)
Calcium-based solutions	- Can significantly increase the calcium´s content of the final product - Delays aging or ripening of fruits and vegetables - Reduces post-harvest decay	- Bitterness and off-flavours associated with calcium chloride	Limited efficacy as antimicrobial agent - Calcium lactate, as fresh-cut lettuce and carrots sanitizer, showed similar effectiveness to chlorine in reducing and keeping the microbial load	(Kitis, 2004, Ölmez and Kretzschmar, 2009)
Ozone	<u>Aqueous</u> - High antimicrobial activity - Extends storage life of fresh non-cut products - Effective at low concentrations and short contact time - Broad spectrum - Good penetration ability - Effectiveness against protozoa reported	<u>Aqueous</u> - Possible deterioration of produce flavour and colour - Can cause produce physiological injury and loss of antioxidant constituents - Unstable, very highly reactive - Possible human toxic effects in processing facilities	<u>Aqueous</u> - Effective against a variety of postharvest pathogens reported on fruits and vegetables	(Alexandre et al., 2011a, Alexandre et al., 2011b, Anino et al., 2006, Conway et al., 1992, Martin-Diana et al., 2005b, Picchioni et al., 1996)

Table 2 (continued). Chemical methods used for the preservation of minimally processed vegetables and fruits

Method	Advantages	Limitations	Effectiveness	References
Ozone (continued)	- Generally recognized as safe (GRAS) - No hazardous DBP formation - Decomposes to nontoxic products - Does not leave hazardous residues on food - Lower running cost Gaseous - Higher antimicrobial activity than in solution - Acceptable food quality - Effective against spoilage and pathogenic microorganisms	- Corrosive to equipment - Requires on-site generation - Requires monitoring in indoor applications - Higher initial investment cost Gaseous - Ozone gas is hazardous so must be contained and destroyed - Toxic and reactive - Can damage plant tissues - Product quality changes may occur -Control of O_3 leakage required	Gaseous - More effective than the solution form	
Electrolysed water (EW)	- Inactivates several pathogenic and spoilage microorganisms - Neutralises harmful substances such as cyanides and ammonium	- Reduces quality on fresh-cut vegetables	- Has strong bactericidal effect on pathogenic and spoilage microorganisms of minimally processed vegetables	(Guzel-Seydim et al., 2004, Habibi and Haddad, 2009, Karaca and Velioglu, 2007)

1.7.2. Physical methods

Some physical methods are also available for reducing the microbiological load of produce. An overview of the major advantages, limitations and effectiveness for each physical method is present in Table 3.

1.7.2.1. Modified packaging

The simple form is vacuum packaging (VP), in this the products are packed in a low O_2 permeable film, the air is evacuated and the package is sealed (Arvanitoyannis, 2012).

1.7.2.1.1. Modified atmosphere packaging (MAP)

Modified atmosphere packaging (MAP) involves the modification of the internal atmosphere composition of a package by reducing the amount of oxygen (O_2) and replacing it by carbon dioxide (CO_2) and/or nitrogen (N_2). This process intends to extend the post-harvest life of whole and pre-cut commodities by reducing their respiration rate and the production of ethylene, minimizing metabolic activity, delaying enzymatic browning, and retaining visual appearance (Cui et al., 2009). The gases re-balancing can be achieved either using active or passive techniques inside a package made of various types and/or combinations of films (Saxena et al., 2008). A drawback of this method is related with the levels of CO_2 generated inside the packages that can not only inhibit the aerobic spoilage microorganisms, but can also allow or even stimulated pathogens growth (Rodriguez-Aguilera et al., 2009, Rosa et al., 2007).

1.7.2.1.2. Active and intelligent packaging

In the last decades, one of the most innovative developments in the area of food packaging is the active and intelligent (A&I) packaging, based on deliberate interactions with the food or food environment (Restuccia et al., 2010).

Active packaging refers to the incorporation of certain agents into packaging systems (whether loose within the pack, attached to the inside of packaging materials or incorporated within the packaging materials themselves) to improve food quality and safety and therefore extend their shelf life (Dainelli et al., 2008, Kerry et al., 2006). The nature of the active agents that can be added is very diverse (organic acids, enzymes, bacteriocins, fungicides, natural extracts, ions, ethanol, etc.) as well as the nature of the materials into which they are included (papers, plastics, metals or combinations of these materials) (Dainelli et al., 2008, Restuccia et al., 2010).

The wide diversity of active packaging devices has specific applications to individual food products for which the shelf-life can be extended substantially, so long as the food's unique spoilage mechanisms are understood and controlled. Many studies can be found, regarding applications of active packaging to food industry (Bolumar et al., 2011, Cruz-Romero et al., 2013).

The purpose of intelligent packaging is to give indication on, and to monitor, the freshness of the food. Substances responsible for the active or intelligent function can be contained in a separate container, for instance in a small paper sachet or as substances directly incorporated in the packaging material (Arvanitoyannis, 2012, Dainelli et al., 2008).

Intelligent packaging gives information to the manufacturer, retailer and consumer based on its ability to sense, detect, or record external or internal changes in the product's environment. It can be used to check the effectiveness and integrity of active packaging systems (Arvanitoyannis, 2012, Kerry et al., 2006).

1.7.2.1.3. Nanocomposite packaging

Currently the food industry uses packages that are non-degradable, generating environmental problems. Several biopolymers have been exploited to develop materials for eco-friendly food packaging, however their poor mechanical and barrier properties have limited their use (de Azeredo, 2009, Duncan, 2011, Roy et al., 2012).

The use of fillers with at least one nanoscale dimension (nanoparticles) produces nanocomposites. These have larger surface area, which favors the filler–matrix interactions and the performance of the resulting material. Recently, using nanocomposite

materials improved fundamental characteristics of food-packaging materials such as strength, barrier properties, antimicrobial properties, and stability to heat and cold (Rhim et al., 2013).

Some of the applications associated with nanotechnology include the development of new food-packaging materials with improved mechanical, barrier and antimicrobial properties (de Azeredo, 2009, Duncan, 2011, Roy et al., 2012).

Nanoscale technologies can be a promising technique to improve produce packaging. However, more studies are required before their application in food industry.

1.7.2.2. Irradiation

Gamma-ray, X-ray, and electron beams are called ionizing radiations, because they are capable of producing ions, electronically charged atoms or molecules. They have the same mechanisms in terms of their effects on foods and microorganisms. The principal target of ionizing radiation is water that produces free radicals, which react, destroy or deactivate bacterial components (Allende et al., 2006b, Rico et al., 2007, Soliva-Fortuny and Martin-Belloso, 2003). Low-dose irradiation is applied to fresh fruits and vegetables to delay produce maturation, and is very effective in reducing bacterial, parasitic, and protozoan pathogens (Lu et al., 2005, Prakash et al., 2000).

This treatment has been approved by the FDA for use on fruits and vegetables at a maximum level of 1.0 kGy (Parish et al., 2003). However, FDA evaluated a petition, filed by the Food Irradiation Coalition, asking for the use of irradiation to enhance safety of fresh-cut produce at doses up to 4.5 kGy. In response, they ruled that only for iceberg lettuce and spinach shelf-life extension an up to maximum dose of 4.0 kGy can be used. The use of ionizing radiation on the remaining foods included in the petition remains under review (FDA, 2008).

1.7.2.3. Ultraviolet light (UV)

Ultraviolet radiation is classified according to wavelength: UV-A, also known as near-ultraviolet radiation, ranges from 315 to 400 nm; UV-B, mid-range UV, from 280 to 315 nm; and UV-C, far-UV, from 100 to 280 nm (Prakash et al., 2000).

UV-C is the most common applied to fresh fruits and vegetables, since it acts directly or indirectly as an antimicrobial agent. UV-C can cause direct bacterial DNA damage or may induce resistance mechanisms against pathogens in different fruits and vegetables. Low doses of UV-C radiation (254 nm) also reduce decay of a wide range of fruits and vegetables when applied after harvest (Ben-Yehoshua and Mercier, 2005).

1.7.2.4. Pulsed light (PL)

An alternative technology to UV light is Pulsed light (PL) also known as High Intensity light pulse (HILP) (Palgan et al., 2011).

This treatment is rapid and effective on microbial inactivation in solid and liquid foods and involves a wide broad-spectrum light in the wave length range of 100 -1100 nm. Pulsed light kills microorganisms using short time high frequency pulses of an intense broad spectrum, rich UV-C light. Explanations for its mechanism of action have been given in terms of structural changes of microbial DNA, comparable to the effect caused by continuous ultraviolet sources, but additional mechanisms seem to be involved (Pataro et al., 2011, Vicente et al., 2005).

Since the PL decontamination effect seems to be dependent on microbial light absorption, certain food components could also absorb the effective wavelengths and decrease the efficiency of this treatment (Ramos-Villarroel et al., 2012).

Current literature on vegetables and fruit decontamination with PL is scarce (Palgan et al., 2011), therefore more data is necessary to evaluate the suitability of this technology.

1.7.2.5. High pressure processing (HPP)

High pressure processing is a method where food is subjected to elevated pressures (in the range of 100–1000 MPa) to achieve microbial and enzymatic inactivation, without the degradation in flavour and nutrients associated with traditional thermal processing.
HHP processing can increase chemical or microbial stability and make desirable textural changes in food products. These achievements will depend on pressure, treatment time, and types of enzymes and/or microorganisms (Guerrero-Beltrán et al., 2005).
As the process can be operated at ambient or even chill temperatures, there is little heat damage to nutrients or natural flavours and colours, which results in high quality products. As shown in several studies, HPP can be suitable for fruit and vegetables products processing. This treatment provides high quality food with higher safety and extended shelf-life, while maintaining similar characteristics to fresh products (Guerrero-Beltrán et al., 2005, Laboissière et al., 2007, Schlüter et al., 2009).

1.7.2.6. Ultrasound

The use of ultrasound within the food industry has been a subject of research and development for many years. Power ultrasound (US) has emerged as an alternative processing technology to food conventional thermal approaches (Oey et al., 2008, Rico et al., 2007). Ultrasound is used at frequencies in the range of 20–100 kHz and requires the presence of a liquid medium for power transmission. On its own, US is not significantly effective on decreasing high load microbial contamination (Alexandre et al., 2012a, Sagong et al., 2011). Due to this reason, this treatment has been used in combination with aqueous sanitizers (e.g. organic acids, chlorine, and chlorine dioxide), showing better results (Cao et al., 2010). The US potential is to improve the aqueous sanitizer's effectiveness by enhancing the penetration of these solutions to inaccessible sites (hydrophobic pockets and folds in leaf surfaces on fruits and vegetables) (Seymour et al., 2002).

1.7.2.7. Cold plasma

An emerging antimicrobial technology for decontaminating infected surfaces is the use of non-thermal ionized gases (cold gas plasmas). Briefly, plasma is composed of gas molecules, which have been dissociated by an energy input. It is constituted by photons, electrons, positive and negative ions, atoms, free radicals and excited or non-excited molecules that, in combination, have the ability to inactivate microorganisms (Fernández et al., 2012).

This flexible sanitizing method uses electricity and a carrier gas, such as air, oxygen, nitrogen, or helium. The primary modes of action are due to UV light and reactive chemical products of the cold plasma ionization process (Niemira, 2012).

In food processing, the direct application of cold plasma as well as semi-direct or indirect treatment with thermal plasma is of interest as these can be used to treat the food at low temperatures (<70ºC). For applications in the food sector, preference should be given to processes carried out at atmospheric pressure (e.g. plasma jet, dielectric barrier discharges) because they allow continuous process control and do not accelerate undesirable phase transitions, compared to applications at reduced pressure ($p < 1013$ mbar) or low pressure ($p < 10$ mbar) (Schlüter et al., 2013).

Studies on produce had shown that cold plasma is highly effective on the removal of surface human pathogens, such as *E. coli* O157:H7 and *Salmonella* spp. (Fernández et al., 2013, Misra et al., 2011, Niemira, 2012, Schlüter et al., 2013, Wang et al., 2012).

The degree of inactivation can be affected by the type of microorganisms, the inactivation medium, number of cells, operating gas mixture, gas flow, and physiological state of cells, among others (Bermúdez-Aguirre et al., 2013).

There is scarce information about physicochemical changes that might occur in the product due to the interaction of charged species from plasma with the food components (Bermúdez-Aguirre et al., 2013, Knorr et al., 2011, Surowsky et al., 2013).

Table 3. Physical methods used for the preservation of minimally processed vegetables and fruits

Method	Advantages	Limitations	Effectiveness	References
Modified atmosphere packaging (MAP)	- Extends storage life of the fresh produce by 50-400 % - In general, fresh-cut products are more tolerant to higher CO_2 concentrations than the intact product - Reduced economic losses - Provides a high-quality product - Odourless and convenient packages - Sealed packages can act as barriers to further product recontamination - Delay of ripening - Improved presentation, clear view of product and all-around visibility	- Often produces high levels of CO_2 with a consequent development of off-flavours and potential stimulation of pathogens growth - Temperature control necessary - Different gas formulations per product type and target microorganism - Packaging material, and temperature per product and/or the target microorganism(s) - CO_2 dissolving to food could lead to package collapse and increased drip - Plastic films may be environmentally undesirable	- Effective on preserving the quality of fresh and processed vegetables and in reducing the postharvest disease incidence for several fruits and vegetables	(Arvanitoyannis, 2012, Cliffe-Byrnes and O'Beirne, 2002, Cui et al., 2009, Graça et al., 2011, Kim et al., 2000, Rico et al., 2008, Saltveit, 2003)
Active and Intelligent packaging (A&I)	- Food quality and sensorial improvements - Food safety improvement - Delays oxidation - Monitorization of the temperature along transport - Control of respiration rate, microbial growth, and moisture migration - Can be used to check the effectiveness and integrity of active packaging systems	- Difficult to evaluate its safety when compared to the traditional packaging - Can occur migration of substances from the package to the food - Incorrect use of the packaging due to the insufficient labeling - Non-efficacious operation of the A&I packaging - Regulation does not cover the use of this type of packaging - Non-uniformity of international legislation - Labeling requirements	- Efficient on product shelf life improvement - Monitors the integrity and safety of the packed product	(Dainelli et al., 2008, Kerry et al., 2006, Yam et al., 2005)

Table 3 (continued). Physical methods used for the preservation of minimally processed vegetables and fruits

Method	Advantages	Limitations	Effectiveness	References
Nanocomposite packaging	- Environment friendly - Improvement of mechanical properties e.g. strength, modulus and dimensional stability - Low permeability to gases, water and hydrocarbons - Thermal stability - Chemical resistance - Electrical conductivity - Optical clarity in comparison to conventionally filled polymers	- Increased viscosity (limits process ability) - May reduce impact performance - Optical issues - Scarce information on formulation/structure/property relationships	- Development of new food-packaging materials with improved characteristics	(de Azeredo, 2009, de Azeredo, 2013)
Irradiation	- Can be performed at room temperature - Can be conducted after packaging - Delays ripening and senescence of climacteric fruits - Extends shelf-life of produce - Low energy costs	- Acceptance of irradiation by consumers - Produce quality may be affected specially at high doses - Product texture alterations	- Acceptance of irradiation by consumers - Produce quality may be affected specially at high doses - Product texture alterations	(Allende and Artes, 2003, Barakat, 2010, Bidawid et al., 2000, Foley et al., 2002, Goularte et al., 2004, Hagenmaier and Baker, 1997, Hussain et al., 2008, Molins et al., 2001)

Table 3 (continued). Physical methods used for the preservation of minimally processed vegetables and fruits

Method	Advantages	Limitations	Effectiveness	References
Ultraviolet Light (UV)	- Absence of residual toxicity - Equipment relatively inexpensive and easy to use - Can reduce deterioration of the produce - Exposure to UV also induces the synthesis of health-promoting compounds such as anthocyanins and stilbenoids	- Pre-treatment normally necessary - Difficulties in accurately measure the UV dose - Increase produce stress and respiration rate, and induce a lignification-like process - Low penetration depth - Limited application on solid food and opaque surfaces - Can cause off-flavours and colour changes	- Effective in reducing microbiota growth in fruits and vegetables - Germicidal at UV-C interval	(Alexandre et al., 2012c, Neves et al., 2012, Ohlsson and Bengtsson, 2002)
Pulsed Light (PL)	- Rapid and effective on microbial inactivation in solid and liquid foods - Few residual compounds - Medium cost - Low energy input	- Food composition affects the efficacy - Efficacy decreases at high contamination levels - Possible resistance in some microorganisms - Possible adverse chemical effects	- Inactivates spoilage and pathogenic microorganisms	(Choi et al., 2010, Gómez-López et al., 2007b, Guerrero-Beltrán et al., 2005, Oms-Oliu et al., 2010)
High Pressure Processing (HPP)	- Microbial and enzymatic inactivation - No degradation in flavour and nutrients - No evidence of toxicity - Positive consumer appeal - Uniformity of treatment throughout food	- Affects porous integrity - Expensive equipment - Foods should have approx. 40 % free water for antimicrobial effect	- Effective in inactivating most vegetative pathogenic and spoilage microorganisms at pressures above 200 MPa	(Chawla et al., 2011, Considine et al., 2008, Guerrero-Beltrán et al., 2005)

Table 3 (continued). Physical methods used for the preservation of minimally processed vegetables and fruits

Method	Advantages	Limitations	Effectiveness	References
Ultrasound (US)	- Enhance the penetration of solutions to inaccessible sites - Heat transfer rate increased - Reduction of process times and temperature	- Needs to be combined with other process to be effective - Complex due to difficulty to scale-up - Changes on food structure and texture - Penetration affected by solids and air in product	- Effective against common food bacteria pathogens - Also effective against vegetative cells, spores and enzymes	(Alexandre et al., 2012a, Cao et al., 2010, Chemat et al., 2011, Mukhopadhyay and Ramaswamy, 2012, Sagong et al., 2011)
Cold Plasma	- High efficiency - Low impact on the internal product matrix -No residues -Resource-efficient -Can be used on vegetables tissues surfaces - No 'shadow effects' (all product is treated) - Could be included as part of the packaging process	- Scarce information about the mechanism of inactivation - Physicochemical changes in the product may occur - Inactivation is affected by type of microorganisms, inactivation medium, number of cells, operating gas mixture, gas flow, and physiological state of cells - Scarce information about interactions with the food or packaging materials - Scarce information about the stability of the plasma for large-scale operation	- Inactivation of *E. coli* O157:H7, *Salmonella*, *S. aureus* and *L. monocytogenes* by 1.5-3.7 log CFU/cm^2	(Bermúdez-Aguirre et al., 2013, Critzer and Doyle, 2010, Fernández et al., 2013, Fernández and Thompson, 2012, Knorr et al., 2011, Perni et al., 2008, Surowsky et al., 2013)

1.8. Biological Methods

In an attempt to prevent growth of pathogens and spoilage microorganisms on produce, research on the application of biocontrol agents has been made. This method is known as biopreservation and consists on extension of the shelf-life and improvement of food safety using microorganisms and/or their metabolites. Some particular microorganisms that have been studied as possessing an antagonistic effect on pathogens are the lactic acid bacteria (LAB). This bacterial group is naturally present in food products and studies suggested that when LAB are applied to produce surfaces they are strong competitors for physical space and nutrients, and/or may produce a wide range of antimicrobial metabolites such as organic acids, hydrogen peroxide, diacetyl and bacteriocins that negatively affect pathogens (Sagong et al., 2011). Bacteriocins are "generally recognized as safe" (GRAS) and have been commonly employed in combination with other food additives as protective agents in fresh-cut produce (Rodgers, 2001, Rodgers, 2008).
Therefore, biopreservation is a promising innovative way of extending the shelf-life of fresh fruits and vegetables, and reducing microbial hazards (Settanni and Corsetti, 2008). Thus, further research on microbial interactions as a pathogens control mitigation strategy in produce is needed.

1.9. Combined Methods

Hurdle technology refers to a combination of different of the above mentioned preservation techniques that supplement and enhance each other. The most important hurdles commonly used are based on storage temperatures, water activity, pH, redox potential, modified atmosphere, and addition of preservatives (Rahman et al., 2011, Randazzo et al., 2009, Trias et al., 2008a, Trias et al., 2008b). The hurdle technology consists on the use of a sequence of mild treatments (low intensity) to inhibit or inactivate the factors responsible for food spoilage, avoiding the use of single treatments at more

severe conditions. Efficacy of the combined preservation methods is usually dependent upon the types of treatment, type and physiology of the target microorganisms, characteristics of produce surfaces, exposure time and concentration of cleaner/sanitizer, pH, and temperature (Singla et al., 2011). The main goal is to use preservation techniques that prolong storage stability and do not have detrimental effects on the quality attributes of the produce (Parish et al., 2003).

1.10. Conclusions

Most of the techniques reviewed in this paper have not yet been adopted by the fresh-cut and minimally processed fruits and vegetables industry. Chlorine continues to be the most commonly used sanitizer due to its efficacy, cost-effectiveness ratio and simple use. The effectiveness of all of these technologies depends on the microbial sensitivity to the sanitizer agent used and, consequently, variable results are commonly reported by researchers. In part, the lack of efficiency can be attributed to the disinfectant inaccessibility to structures and tissues that support the growth of microbial flora. Further investigation on specific pathogens/produce combinations is needed.

It can be concluded that there are many different technologies to reduce/eliminate the microorganisms present in fresh-cut fruits and vegetables. The proper use of these techniques will allow an increase safety of the minimally processed products. However, none of the sanitizing methods can control all the parameters that maintain the quality and shelf-life of MPFVs. Therefore, additional studies using combined methods or using competitive microbiota to extend and enhance the safety of this kind of products are crucial.

1.11. Acknowledgements

This work was supported by National Funds from FCT – Fundação para a Ciência e a Tecnologia through project PEst-OE/EQB/LA0016/2011.

Financial support for authors Ramos B., Miller F.A. and Brandão T.R.S. was provided by FCT and Fundo Social Europeu (FSE) through fellowships SFRH/BD/42169/2007, SFRH / BPD / 65041 / 2009 and SFRH / BPD / 41419 / 2007, respectively.

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Chapter 2

Applications of protective cultures, bacteriocins and bacteriophages to fresh and minimally processed vegetables: a review.

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Submitted to *Food Reviews International (February. 2016).*

2.1. Abstract

The interest on minimally processed vegetables prepared without chemical preservatives has prompted the quest for biopreservation or biological control (biocontrol) approaches. Protective cultures of lactic acid bacteria have been developed over the last few decades to increase the safety and shelf-life of these products. Bacteriocins have been widely recognized as natural food biopreservatives, and the latest advances in bacteriocin biology opened new fields to explore. Recently, the use of bacteriophages has been considered, with promising perspectives.

This review provides an overview of the current and novel applications of protective cultures, bacteriocins and bacteriophages along fresh and minimally processed vegetables processing.

Keywords: Vegetables, lactic acid bacteria, protective cultures, bacteriocins, bacteriophages

2.2. Introduction

The natural microbiota of raw vegetables are usually non-pathogenic for humans and may be present at the time of consumption (Ahvenainen, 1996, Food and Drug Administration, 2008). However, during growth, harvest, transportation and further processing and handling, produce can be contaminated with pathogens from human, animal or environmental sources (Ahvenainen, 1996, Brandl, 2006, Froder et al., 2007). As a result, these products can be a vehicle of transmission of bacterial, parasitic and viral pathogens, capable of causing human illness.

Nowadays, a wide range of technologies are available to eliminate pathogens from the food chain (Ramos et al., 2013). However, pathogens may be more resistant to the

decontamination procedures than the indigenous microbiota, and by removing this group of microorganisms, a natural barrier for pathogenic growth is also removed. As a result, disinfection may provide conditions which favour survival and growth of the pathogens. Furthermore, reduction of the natural microbiota could be risky if further hurdles are not applied to the produce, especially if the produce is contaminated with a pathogen after processing (Allende et al., 2008). Due to the pathogen mechanisms to survive, and the increasing consumption of minimally processed and ready to eat vegetables, outbreaks associated with produced are still frequent (Gandhi and Chikindas, 2007, Naghmouchi et al., 2007, Ramos et al., 2014, Ramos et al., 2013). *Salmonella* spp., *Escherichia coli*, *Shigella* spp. and *Listeria monocytogenes* are amongst the major pathogens associated to outbreaks caused by the consumption of contaminated vegetables. The produce most associated with outbreaks is salad, since it has all kind of mixed vegetables (Ramos et al., 2013). In addition, WHO categorized lettuce and salads (all varieties), leafy vegetables (spinach, cabbage, raw watercress) and fresh herbs of highest priority in terms of fresh produce safety from a global perspective (FAO/WHO, 2008, Goodburn and Wallace, 2013).

An increasing number of consumers prefer minimally processed foods, prepared without chemical preservatives. Many of these ready-to-eat and novel food types represent new food systems with respect to health risks and spoilage association. Relying on improved understanding and knowledge of the complexity of microbial interactions, one alternative approach is biopreservation or biological control (biocontrol) to prevent growth of pathogens and spoilage microorganisms in produce (Holzapfel et al., 1995, Kostrzynska and Bachand, 2006, Ramos et al., 2013).

By biopreservation, storage life is extended and food safety improved through the use of native microbiota and/or their metabolites. Different mechanisms, like production of inhibitory compounds, competition for nutrients, space or even colonization sites, are responsible for pathogen inhibition by biocontrol agents. The use of protective cultures of generally recognized as safe (GRAS) microorganisms, such as lactic acid bacteria (LAB), have been developed over the last few decades to increase the safety and shelf-life of fresh and minimally processed vegetables (Engelhardt et al., 2015, Ramos et al., 2013). In addition, several other microorganisms from the natural microbiota, including

strains of *Pseudomonas fluorescens, Pseudomonas syringae*, *Pseudomonas viridiflava, Gluconobacter asaii* and *Enterobacter asburie* have been proposed as biocontrol agents in these foods (Siroli et al., 2015a).

Bacteriocins, ribosomally synthesized antimicrobial peptides, are able to kill or inhibit the growth of other bacteria and are considered to be safe natural biopreservatives. The direct application of bacteriocins in fresh cut products has been tested in recent years with promising results (Allende et al., 2007, Molinos et al., 2005, Randazzo et al., 2009, Siroli et al., 2015a).

Bacteriophage (phage) prophylaxis is also a possible natural method to be used as a biopreservative. Phages are bacterial viruses that invade specific bacterial cells, disrupt bacterial metabolism, and cause the bacterium to lyse without compromising the viability of other flora in the habitat. They are the most abundant microorganisms in our environment and are present in high numbers in water and foods. Promising results using phage biocontrol have been reported for several pathogens, including *Salmonella* spp., *L. monocytogenes* and *E. coli* O157:H7 (Oliveira et al., 2015).

This is an overview of all past year's research to find effective biopreservation approaches. This review particularly focuses on the use of protective cultures, bacteriocins and bacteriophages to control pathogens in fresh and minimally processed vegetables.

2.3. Protective Cultures

A distinction can be made between protective and starter cultures. In fact, the same culture can be used with different purposes under different conditions. For a protective culture the antimicrobial activity is the key effect whilst the metabolic activity and technological potential (e.g. exopolysaccharides production and contribution to flavour development) is a secondary effect. For a starter culture the functional objective is the inverse (Holzapfel et al., 1995). In short, antagonistic cultures that are only added to inhibit pathogens and/or prolong the shelf life, while changing the sensory properties of the food product as little

as possible, are termed protective cultures. Protective cultures are mainly selected due to their potential to produce bioactive metabolites with antimicrobial activity, which must not alter or negatively interfere with the food matrix (Matamoros et al., 2009).

In order to grow in the fresh and minimally processed vegetables, protective cultures should tolerate the naturally occurring antimicrobial compounds present in many vegetables and be able to efficiently use the nutrients available (Table 1). Temperature is also a key factor, as these kind of food products relay on the cold storage to maintain its quality and safety. So, protective cultures should be able to grow and to cause their inhibitory activity on the target microorganisms at low temperatures (Gálvez et al., 2012b).

Table 1. Desirable properties of protective cultures to be applied as biopreservation agent to fresh foods (adapted from Holzapfel et al. (1995))

Target Microorganism
High inhibitory activity
Antimicrobial activity at low temperatures
Competitiveness against autochthonous organisms
Health
No health risk
No production of toxins
No production of biogenic amines or other metabolites detrimental to health
Non pathogenic
Product
Adapt to product / substrate
No negative sensory effects
No negative nutritional effects
Predictability of metabolic activity under given conditions
Specific enzymatic activity
Function as ‘indicator’ under abuse conditions
Compatible with other control systems, and therefore can be applied together (hurdle technologies)

Lactic acid bacteria are particularly interesting candidates for biological control. They are naturally present in food and are often strong competitors. Their use and the use of their metabolites are generally recognized as safe (GRAS) and benefit from the healthy image of many dairy products (Deegan et al., 2006, Holzapfel et al., 1995, Zacharof and Lovitt, 2012).

2.3.1. LAB cultures and antimicrobial mechanisms

Application of a protective culture for antimicrobial protection of food should be considered only as an additional measure to good manufacturing, processing, storage and distribution practices. Lactic acid bacteria cultures have been used to preserve meat, fish, dairy products and fermented vegetables and fruit juices (Galvez et al., 2007, Rodgers, 2001, Siroli et al., 2015a). In addition, protective cultures of LAB have been developed over the last decades to increase the safety and shelf-life of fresh and minimally processed vegetables (Siroli et al., 2015a, Siroli et al., 2015b, Trias et al., 2008a, Vescovo et al., 1996). The biocontrol potential of protective cultures is affected by numerous factors as shown in Table 2.

The preservative effect of LAB is due to the production of one or more active metabolites, such as organic acids (lactic, acetic, formic, propionic acids) that intensify their action by reducing the pH of the media, and other substances, like fatty acids, acetoin, hydrogen peroxide, diacetyl, antifungal compounds (propionate, phenyl-lactate, hydroxyphenyl-lactate, cyclic dipeptides and 3-hydroxy fatty acids), bacteriocins (nisin, reuterin, reutericyclin, pediocin, lacticin, enterocin and others) and bacteriocin-like inhibitory substances – BLIS (Galvez et al., 2007, Holzapfel et al., 1995, Matamoros et al., 2009, Reis et al., 2012).

Table 2. Factors affecting protective culture effectiveness in fresh foods (Galvez et al., 2007, Rodgers, 2001)

Factor	Impact
Increase in biopreservation agent inoculation level	Reduction of the time needed to produce the inhibitory metabolite(s) Excessive inoculums can inhibit culture growth and bacteriocin production Physiological state Microbial interactions Microbial diversity
Increase in storage temperature	Increases the growth rate Increases the metabolic rate of the vegetables Can reduce bacteriocin producing capacity and bacteriocin activity Can reduce the target microorganism sensitivity
Biopreservation agent	Antibacterial spectrum Adaption to food environment Sensitivity to food composition (nutrients, ingredients, additives, etc.) Sensitivity to food environment (pH, temperature, redox potential time, a_w, O_2, CO_2, etc.) Rate of inhibitory metabolite(s) production Growth and bacteriocin production temperature Possible physicochemical and sensorial alterations of the food products Potential to spoil the food Possible health benefits/hazards Resistance to processing Interaction with other hurdles
Type of food	Growth and inhibitory substances diffusion rate Indigenous microbiota can produce its own inhibitory effect Antimicrobial substances can have a synergetic effect with the antibiosis Growth and bacteriocin-production promoting substances can enhance the antagonistic effect

2.3.1.1. Organic Acids

Organic acids such as lactic, acetic and propionic acids are end products of LAB fermentation. They have antimicrobial activity towards Gram (+) and Gram (-) bacteria due to the interference in the potential of the cell membrane, inhibition of the active transport, pH reduction and to the inhibition of metabolic functions (O'Bryan et al., 2015, Reis et al., 2012).

2.3.1.2. Hydrogen Peroxide (H_2O_2)

Production of H_2O_2 by LAB can prevent the growth of foodborne pathogens, and can also be beneficial in food preservation. The antimicrobial effect derives from the increasing of the membrane permeability, by denaturation of enzymes and peroxidation of the membrane lipids. Lactic acid bacteria that produce H_2O_2 have been shown to inhibit the growth of pathogenic microorganisms at refrigeration temperatures (Reis et al., 2012).

2.3.1.3. Diacetyl

Some LAB produce diacetyl from the excessing pyruvate. Diacetyl is a nonpolar, volatile diaketone that can inhibit Gram (-) bacteria by obstructing the arginine utilization (O'Bryan et al., 2015).

2.3.1.4. Bacteriocins

Bacteriocins are defined as a group of heterogeneous, bioactive peptides or proteins with antimicrobial activity against other bacteria (Beshkova and Frengova, 2012). A substantial number of Gram (+) and Gram (-) bacteria produces bacteriocins during their growth. Usually they have a low molecular weight (rarely over 10 kDa) and are active against strains of species related to the producing bacteria (Zacharof and Lovitt, 2012). The fact that bacteriocins, active against numerous foodborne and human pathogens, are produced by GRAS microorganisms and are readily degraded by proteolytic host systems,

makes them attractive candidates for biotechnological applications (Settanni and Corsetti, 2008).

Further aspects of bacteriocins will be discussed below.

2.3.2. Applications of protective cultures to fresh and minimally processed vegetables

The first attempts to control pathogens in fresh produce using biological agents were based on bacteriocinogenic LAB strains. However, other bacteria have been demonstrating the potential to be effective biopreservative cultures. A summary of bacteriogenic culture applications in fresh and minimally processed vegetables is presented in Table 3.

2.3.2.1. Lactic acid bacteria (LAB)

The potential of bacteriocin producing LAB to inhibit microbial populations of ready to eat salads was demonstrated by Vescovo et al. (1995). In particular, coliforms and enterococci were strongly reduced or eliminated from the product after inoculation with LAB cultures (Vescovo et al., 1995). From five LAB strains tested in salads, *Lactobacillus casei* IMPCLC34 was the most effective in reducing the total mesophilic bacteria and the coliform group as well as *Aeromonas hydrophila*, *Salmonella typhimurium* and *Staphylococcus aureus*, while the *Listeria monocytogenes* counts remained constant (Vescovo et al., 1996).

The bacteriocin producing *Lactococcus lactis* subsp. *lactis,* isolated from bean sprouts, was able to reduce *L. monocytogenes* counts on ready to eat Caesar salad by 1 to 1.4 logs during storage for 10 days at 7 and 10 ºC (Cai et al., 1997).

Torriani et al. (1997) showed that the addition of *Lb. casei* IMPC LC34 and of 3% of its culture permeate to mixed salads reduced the total mesophilic bacteria counts, and suppressed coliforms, enterococci, and *A. hydrophila* after 6 days of storage at 8 °C.

Torriani et al. (1999) revealed the ability of *Lactobacillus plantarum* IMPC LP4 to extend the shelf life of shredded carrots, by controlling the growth of their microbiota, particularly *Leuconostoc* spp..

The inhibitory activity of *Lac. lactis* against *L. monocytogenes* was assessed on alfalfa sprouts. When the co-inoculation onto the seeds was made at the beginning of the sprouting process, the maximum inhibition of *L. monocytogenes* was approximately 1 log unit (Palmai and Buchanan, 2002). The authors reported a decrease in the effectiveness of the biopreservation agent in real systems compared with model systems, by the action of the sprout microbiota.

In a model reproducing the characteristics of fresh vegetables, Scolari et al. (2004) showed that although the growth of *Lb. plantarum* and *Staph. aureus* was affected by temperature, the pathogenic strain independently of its inoculum size, was always inhibited by *Lb. plantarum*. They suggested that a proper combination of specific LAB and storage temperature should improve the safety of the vegetable products.

Scolari and Vescovo (2004) performed various tests in scarola salad leaves that indicated the remarkable inhibitory effect of *Lb. casei* towards *Staph. aureus*, *A. hydrophila*, *E. coli* and *L. monocytogenes*.

One of the largest screenings for antagonistic bacteria was performed by Trias et al. (2008b) among seven hundred samples of fresh fruit and vegetables. Lactic acid bacteria were isolated and tested for the capacity to inhibit *E. coli, L. monocytogenes, Pseudomonas aeruginosa, S.* Typhimurium*,* and *Staph. aureus* on Iceberg lettuce cuts. The selected strains, predominantly belonged to *Leuconostoc* spp. and *Lb. plantarum*, and a few corresponded to *Weissella* spp. and *Lb. lactis*. The antagonist's strains reduced the *S.* Typhimurium and *E .coli* counts by 1 to 2 log CFU/wound or g, and inhibited the growth of *L. monocytogenes* in lettuce cuts. On the other hand, the strains did not cause negative effects on the general aspect of lettuce tissues.

In a subsequent work, Trias et al. (2008a) tested ten *Leuconostoc mesenteroides* and one *Leuconostoc citreum* strains isolated from fresh produce for their antagonistic capacity against *L. monocytogenes*. The inhibition effect was due to organic acids, hydrogen peroxide and bacteriocins production. In this study, the effect of relative dose of pathogen

and LAB on *L. monocytogenes* inactivation was assessed, and the importance of the protective culture inoculum on biopreservation approaches was emphasized.

A study focusing on the inhibition of *L. monocytogenes,* from minimally processed kale, revealed that *Pediococcus acidilactici* CCA3, a strain isolated from kale, was able to inhibit the pathogen (2.3 log units at 15 ºC) and did not alter the product sensorial characteristics during the shelf life period (Costa et al., 2009).

Yang et al. (2012) revealed the potential of bacteriogenic LAB isolated from dairy products to control *Listeria innocua* on fresh cut onions.

The capacity of commercial LAB food antimicrobials to inhibit pathogens in produce has also been demonstrated. Bovamine® (Nutrition Physiology Corporation, Guymon, OK) effectively inhibited *E. coli* O157:H7 on baby spinach surfaces kept at refrigeration temperature (Gragg and Brashears, 2010). Similarly, LactiGuard™ (Guardian Food Technologies, LLC, Overland Park, KS), applied to spinach, reduced by 1.4 and 1.1 log units the counts of *E. coli* O157:H7 and *Clostridium sporogenes*, respectively (Brown et al., 2011). In another experiment, the application of LactiGuard™ significantly reduced *E. coli* O157:H7 and *Salmonella enterica* populations on spinach by 1.6 and 1.9 log CFU/g, respectively (Calix-Lara et al., 2014).

Valerio et al. (2013) reported the capacity of *Lb. paracasei* LMGP22043 to inhibit the growth of *L. monocytogenes*, *S. enterica* subsp. *enterica* and *E. coli* in ready to eat artichoke products.

Siroli et al. (2014) revealed the effectiveness of the nisin producing strain *Lac. lactis* CBM21, inoculated in the washing solution of minimally processed lamb's lettuce, to inhibit *L. monocytogenes*, *E. coli* and the total mesophilic species and significantly increase the product shelf-life. Moreover, Siroli et al. (2015b) showed that applying *Lb. plantarum* V7B3 and *Lb. casei* V4B4 to lettuce during the washing phase can increase its safety and shelf-life. In fact, *Lb. plantarum* V7B3 increased *E. coli* death kinetics and reduced the viability of *L. monocytogenes.* In addition, combining the selected strains with natural antimicrobials produced a further increase in the shelf life of these products, without affecting the organoleptic qualities.

Recently, Ramos et al. (Submitted to Food Microbiology) demonstrated the high capacity of *Pediococcus pentosaceus* DT016, previously isolated from lettuce (Ramos et al.,

2016), to control *L. monocytogenes* proliferation on fresh lettuce, rocket salad, parsley and spinach kept under cold storage.

2.3.2.2. Other bacteria

Gram (-) bacteria dominates the microbiota of most vegetables. They have a high potential to be use as biopreservation agents to reduce pathogen growth and survival in produce. These organisms have the advantage of being part of the natural microbial community that is already established on the target produce, which may facilitate their colonization and survival when applied in appropriate numbers (Gálvez et al., 2012b, Ramos et al., 2013, Siroli et al., 2015a).

Carlin et al. (1996) found that *P. fluorescens* inhibited the growth of *L. monocytogenes* by 1 log unit, on endive leaves stored at refrigeration (10 ºC).

Liao and Sapers (1999) in a study on potato tuber slices, verified that *P. fluorescens* and *P. viridiflava* have antagonist activity towards *L. monocytogenes* growth.

The native microbiota of green bell peppers, Romaine lettuce, baby carrots, alfalfa and clover were screened for their ability to inhibit the growth of *Salmonella* Chester, *L. monocytogenes*, *E. coli* and *Erwinia carotovora* subsp. *carotovora*. Six isolates with the capacity for inhibition of at least one pathogen were selected and identified as *Bacillus* spp. (three strains), *P. aeruginosa* (one strain), *P. fluorescens* and a yeast. The application of *P. fluorescens* and the yeast to green pepper disks resulted in a reduction of *S.* Chester and *L. monocytogenes* populations of 1 and 2 log units, respectively (Liao and Fett, 2001).

Schuenzel and Harrison (2002) screened the microbiota of ready to eat salads for antibacterial activity against *Staph. aureus*, *E. coli*, *L. monocytogenes* and *Salmonella* Montevideo. Of the 1.180 isolates screened for inhibitory activity, 37 (3.22 %) were found to have various degrees of inhibitory activity against at least one pathogen, and several isolates showed inhibitory activity against all four pathogens.

Fluorescent pseudomonads, especially *P. fluorescens*, play an important role in the survival and growth of *Salmonella* spp. in sprouts (Matos and Garland, 2005). In previous trials, *P. fluorescens* isolated from plants have shown inhibitory activity towards a wide

range of spoilage and pathogenic bacteria (Liao, 2006). Fett (2006) found that *P. fluorescens* 2–79 was effective in inhibiting the growth of *S. enterica* on sprouts. In addition, other studies reported that treatment with *P. fluorescens* 2–79 reduced the growth of salmonellae by 2–3 log units on alfalfa seeds (Liao, 2008), and reduced other human pathogens on bell pepper disks (Liao, 2009).

Weiss et al. (2007) verified that *Pseudomonas jessenii* supressed the growth of *Salmonella* Senftenberg LTH5703 on ready to eat sprouts, and therefore was considered to be used as a protective culture for produce.

The natural microbiota of fresh cut iceberg lettuce and baby spinach was evaluated to isolate and identify antagonist bacteria towards *E. coli* O157:H7. Evidence of naturally occurring microorganisms and of possible antagonistic activity toward *E. coli* O157:H7 on fresh lettuce (295 isolates) and spinach (200 isolates) was documented, displaying that produce microbiota can have inhibitory activities towards foodborne pathogens (Johnston et al., 2009).

Enterobacter asburiae is commonly associated with plants and has been tested as a biocontrol strain for inhibiting the growth of enteric pathogens, such as *Salmonella* and *E. coli* O157:H7 (Cooley et al., 2003). *Ent. asburiae* JX1, isolated from mung bean sprouts, exhibited antibacterial activity against a broad range of *Salmonella* serovars. The antagonistic bacteria in combination with lytic bacteriophages (F01, P01, P102, P700, P800 and FL 41) was tested to control the growth of *Salmonella* spp. on sprouting mung beans and alfalfa seeds. The combination of the biopreservative agents was effective in controlling the pathogen growth on sprouting alfalfa seeds and on mung beans, reducing the pathogen levels by 5.7 to 6.4 log CFU/mL (Ye et al., 2010).

Other authors assessed the effect of *P. fluorescens* on the *E. coli* O157:H7 fate on baby spinach. The biopreservation agent reduced the pathogen loads by 0.5-2.1 log CFU/g of spinach and proved to yield moderate reductions of *E. coli* O157:H7 populations on spinach, when the ratios of *P. fluorescens* to pathogen were similar (Olanya et al., 2013).

Oliveira et al. (2015) isolated and tested *Pseudomonas* spp. strain M309 against *S. enterica*, *E. coli* and *L. monocytogenes* on lettuce disks. M309 strain was highly effective at controlling *S. enterica* and *E. coli* O157:H7 growth on lettuce disks (*in vivo* assay). Furthermore, they tested various biopreservative agents, including M309 strain and

Pseudomonas graminis CPA-7 (Alegre et al., 2013), against *Salmonella* and *L. monocytogenes* on fresh cut lettuce. The addition of M309 strain and CPA-7 strain did not result in a significant reduction of *Salmonella* population. However, CPA-7 strain reduced *L. monocytogenes* numbers in 1.5 log units after 6 days at 10 °C. Results for other biopreservative agents (nisin and bacteriophages) are referred in the corresponding section (2.4. Bacteriocins and 2.5. Bacteriophages).

Table 3. Applications of bacteriogenic cultures to fresh and minimally processed vegetables

Biopreservation agent	**Target microorganism**	**Food**	**Reference**
Pseudomonas fluorescens	*Listeria monocytogenes*	Endive leaves	(Carlin et al., 1996)
Lactobacillus casei IMPCLC34	*Aeromonas hydrophila* *Salmonella typhimurium* *Staphylococcus aureus* *Listeria monocytogenes*	Vegetable salads	(Vescovo et al., 1996)
Lactococcus lactis subsp. *lactis*	*Listeria monocytogenes*	Ready to eat Caesar salad	(Cai et al., 1997)
Lactobacillus casei IMPC LC34	Coliforms Enterococci *Aeromonas hydrophila*	Mixed salads	(Torriani et al., 1997)
Pseudomonas fluorescens *Pseudomonas viridiflava*	*Listeria monocytogenes*	Potato tuber slices	(Liao and Sapers, 1999)
Lactobacillus plantarum IMPC LP4	*Leuconostoc* spp.	Shredded carrots	(Torriani et al., 1999)
Pseudomonas fluorescens and yeast	*Salmonella* Chester *Listeria monocytogenes*	Green pepper disks	(Liao and Fett, 2001)
Lactococcus lactis	*Listeria monocytogenes*	Alfalfa sprouts	(Palmai and Buchanan, 2002)

Table 3 (continued). Applications of bacteriogenic cultures to fresh and minimally processed vegetables

Biopreservation agent	Target microorganism	Food	Reference
Gram (-) microbiota	*Staphylococcus aureus* *Escherichia coli* *Listeria monocytogenes* *Salmonella montevideo*	Model system	(Schuenzel and Harrison, 2002)
Lactobacillus plantarum	*Staphylococcus aureus*	Fresh vegetables model system	(Scolari et al., 2004)
Lactobacillus casei	*Staphylococcus aureus* *Aeromonas hydrophila* *Escherichia coli* *Listeria monocytogenes*	Scarola salad leaves	(Scolari and Vescovo, 2004)
Pseudomonas fluorescens	*Salmonella* spp.	Sprouts	(Matos and Garland, 2005)
Pseudomonas fluorescens 2–79	*Salmonella enterica*	Sprouts	(Fett, 2006)
Pseudomonas jessenii	*Salmonella* Senftenberg	Ready-to-eat sprouts	(Weiss et al., 2007)
Pseudomonas fluorescens 2–79	*Salmonella* spp.	Alfalfa seeds	(Liao, 2008)

Table 3 (continued). Applications of bacteriogenic cultures to fresh and minimally processed vegetables

Biopreservation agent	Target microorganism	Food	Reference
Leuconostoc spp. *Lactobacillus plantarum*, *Weissella* spp. *Lactococcus lactis*	*Salmonella typhimurium* *Escherichia coli* *Listeria monocytogenes*	Iceberg lettuce cuts	(Trias et al., 2008b)
Leuconostoc mesenteroides *Leuconostoc citreum*	*Listeria monocytogenes*	Iceberg lettuce	(Trias et al., 2008a)
Pseudomonas fluorescens 2–79	*Listeria monocytogenes* *Yersinia enterocolitica*	Bell pepper disks	(Liao, 2009)
Pediococcus acidilactici CCA3	*Listeria monocytogenes*	Minimally processed kale	(Costa et al., 2009)
Enterobacter asburiae JX1 alone, or in combination with a Bacteriophage cocktail	*Salmonella* spp.	Mung beans Alfalfa seeds	(Ye et al., 2010).
Bovamine® (commercially available LAB culture product)	*Escherichia coli* O157:H7	Baby spinach	(Gragg and Brashears, 2010)
LactiGuard™ (commercially available LAB culture product)	*Escherichia coli* O157:H7 *Clostridium sporogenes*	Spinach	(Brown et al., 2011)

Table 3 (continued). Applications of bacteriogenic cultures to fresh and minimally processed vegetables

Biopreservation agent	Target microorganism	Food	Reference
Bacteriogenic LAB	*Listeria innocua*	Fresh cut onions	(Yang et al., 2012)
Lactobacillus paracasei LMGP22043	*Listeria monocytogenes* *Salmonella enterica* subsp. *enterica* *Escherichia coli*	Ready to eat artichoke products	(Valerio et al., 2013)
LactiGuard™ (commercially available LAB culture product)	*Escherichia coli* O157:H7 *Salmonella enterica*	Spinach	(Calix-Lara et al., 2014)
Lactococcus lactis CBM21	*Listeria monocytogenes* *Escherichia coli* Total mesophilic species	Minimally processed lamb's lettuce	(Siroli et al., 2014)
Lactobacillus plantarum V7B3 *Lactobacillus casei* V4B4	*Escherichia coli* *Listeria monocytogenes*	Lamb's lettuce	(Siroli et al., 2015b)
Pseudomonas spp. strain M309	*Salmonella enterica* *Escherichia coli* *Listeria monocytogenes*	Lettuce disks (*in vivo* assay)	(Oliveira et al., 2015)
*P*seudomonas *graminis* CPA-7 *Pseudomonas* spp. strain M309	*Listeria monocytogenes* *Salmonella* spp.	Fresh cut lettuce	(Oliveira et al., 2015)

Table 3 (continued). Applications of bacteriogenic cultures to fresh and minimally processed vegetables

Biopreservation agent	Target microorganism	Food	Reference
Pediococcus pentosaceus DT016	*Listeria monocytogenes*	Iceberg lettuce Rocket salad Spinach Parsley	(Ramos et al., Submitted to Food Microbiology)

2.4. Bacteriocins

Bacteriocins are bacterial ribosomally synthesised peptides or proteins with antimicrobial activity, and are generally recognized as "natural" compounds (Galvez et al., 2007, García et al., 2010, Settanni and Corsetti, 2008).

Bacteriocins are bactericidal with some exceptions. Inhibitory activity of the bacteriocin producing strains are mostly confined to Gram (+) bacteria. Most of the bacteriocins described to date act by inserting into the bacterial cytoplasmic membrane (Balciunas et al., 2013, Gálvez et al., 2012a). Other bacteriocins do not interact with the bacterial cytoplasmic membrane, they bind to lipid II and arrest cell wall synthesis (e.g. mersacidin) or act by inhibiting septum formation, halting cell division (e.g. lactococcin 972). Colicins are the most diverse in mode of action, for example pore-formers, DNAses, RNAses, and peptidoglycan synthesis inhibitors (Balciunas et al., 2013, Bennik et al., 1999).

Bacteriocins are often confused in the literature with antibiotics. This would limit their use in food applications from a legal standpoint (Cleveland et al., 2001). They can be distinguished from antibiotics on the basis of synthesis, mode of action, antimicrobial spectrum, toxicity and resistance mechanisms. The major difference is that bacteriocins restrict their activity to strains of species related to the producing species, and particularly to strains of the same species. In addition, bacteriocins are produced in the primary phase of growth, though antibiotics are usually secondary metabolites (Deegan et al., 2006, Settanni and Corsetti, 2008, Zacharof and Lovitt, 2012).

Bacteriocins comprise a very heterogeneous group regarding their primary structure, composition and physicochemical properties (Deegan et al., 2006). A classification into four classes has been proposed by Heng and Tagg (2006) (Table 4). These classes also include several subclasses, according to bacteriocin structure. Class I includes the lantibiotics family. Class II includes small, heat-stable peptide bacteriocins and is by far the largest class among Gram (+) bacteriocins. Of particular relevance for food biopreservation is the potent antilisterial activity displayed by the pediocin-like bacteriocins produced by *Pediococcus* spp. . Class III includes bacteriolytic and non-lytic

large proteins. Class IV includes cyclic peptides. The LAB bacteriocins that have been applied in food biopreservation belong to Class Ia, II and IV (Gálvez et al., 2012a, García et al., 2010, Heng and Tagg, 2006).

Table 4. Bacteriocin classification (according to Heng and Tagg (2006))

Class I-Lantibiotic peptides
Ia-Linear, e.g. nisin, lacticin 481, plantaricin C
Ib-Globular, e.g. mersacidin
Ic-Multi-component, e.g. lacticin 3147, plantaricin W
Class II- Unmodified peptides (<10 kDa)
IIa-Pediocin-like, e.g. pediocin PA-1
IIb- Miscellaneous, e.g. enterocin L50
IIc- Multi-component, e.g. lactococcin G, plantaricin S,
Class III- Large peptides (> 30 kDa)
IIIa- Bacterolytic, e.g. enterolysin A, lysostaphin
IIIb- Non-lyptic, e.g. helveticin J., colicins
Class IV- Cyclic peptides
e.g. AS-48, gassericin A, acidocin B

In recent years, a large number of bacteriocin producing LAB, including *Lactobacillus*, *Lactococcus*, *Leuconostoc* and *Pediococcus* spp., have been isolated from a variety of foods (Abrams et al., 2011, Albano et al., 2007, Cleveland et al., 2001, Gao et al., 2015, Todorov et al., 2011). However, the use of bacteriocins as food additives demands an exhaustive evaluation for toxicological effects before legal acceptance. For that reason, nisin and pediocin PA-1 are the only bacteriocins commercially exploited to date (Sobrino-López and Martín-Belloso, 2008).

2.4.1. Application of bacteriocins to fresh and minimally processed vegetables

Bacteriocins can be incorporated into foods as a concentrated, though not purified, preparation made with food-grade technique. Some desirable properties of the bacteriocins are listed in Table 5.

Table 5. Desirable properties of bacteriocins to be applied as biopreservative agents to fresh foods

Microorganism
Bactericidal mode of action
Acting on the bacterial cytoplasmic membrane
No cross resistance with antibiotics
Feasible genetic manipulation
Health
Non toxic
GRAS status
Inactivated by digestive proteases
Little influence on the gut microbiota
Effective at low concentrations
Product
pH tolerant
High temperature tolerant
Stable at storage conditions
Extend the shelf life
No negative sensory effects
No negative nutritional effects
Low cost
Extra protection during temperature abuse conditions
Reduced risk of pathogenic cross-contamination
Reduce the use of chemical preservatives
Less severe than heat treatments
Marketing of "novel" foods (less acidic, with a lower salt content, and with a higher water content)
Compatible with other control systems, and therefore can be applied together (hurdle technologies)
No medical application

The direct addition of purified bacteriocins obviously provides a more controllable preservative tool in such products. The application of bacteriocins to fresh and fresh cut vegetables has been tested in recent years (Table 6).

Bennik et al. (1999) isolated a bacteriocin producing *Enterococcus mundtii* from minimally processed vegetables, and reported the potential of mundticin as a biopreservative agent applied to mung bean sprouts in a washing step or a coating procedure.

Bari et al. (2005) assessed the effect of nisin and pediocin individually, or in combination with sodium lactate, citric acid, phytic acid, potassium sorbate and EDTA, as potential sanitizers against *L. monocytogenes* on fresh cut cabbage, broccoli and mung bean sprouts. They found that when tested alone all compounds resulted in a reduction between 2-4 log units of *L. monocytogenes* from the vegetables. However, the combination of nisin plus pediocin plus phytic acid was the most effective in reducing the pathogen population.

In a vegetable food model system, the antibacterial efficiency of bacteriocins, from *Lactobacillus* isolates from appam batter (LABB) and vegetable pickle (LABP), and nisin were evaluated, individually and in combination, against *L. monocytogenes* and *Staph. aureus*. The bacteriocin LABB was the most effective in inhibiting the two pathogens, compared to either nisin or LABP, while the combination of LABB with nisin resulted in further reductions of both pathogens (Jamuna et al., 2005).

Molinos et al. (2005) assessed the effect of immersion solutions containing enterocin AS-48 for decontamination of vegetables by *L. monocytogenes*. In particular, treatments with the bacteriocin alone or in combination with chemical preservatives were tested on alfalfa sprouts, soybean sprouts and green asparagus. For the sprouts treated with enterocin AS-48, the *L. monocytogenes* viable counts were reduced below detection limits at days 1 to 7 at 6 ºC and 15 °C, and in green asparagus at 15 °C. Treatment with solutions containing enterocin AS-48 and chemicals, such as lactic acid, sodium lactate, sodium nitrite, sodium nitrate, trisodium phosphate, trisodium trimetaphosphate, sodium thiosulphate, *n*-propyl *p*-hydroxybenzoate, *p*-hydoxybenzoic acid methyl ester, hexadecylpyridinium chloride, peracetic acid, or sodium hypochlorite, reduced *L. monocytogenes* viable counts below the detection limits. Significantly increased antimicrobial activity was found for AS-48

in combination with potassium permanganate, acetic acid, citric acid, sodium propionate, and potassium sorbate.

Allende et al. (2007) tested the effect of washing with bacteriocin solutions (nisin+, coagulin+ and a nisin–/ coagulin+ mixture) on the survival and proliferation of *L. monocytogenes* on fresh cut lettuce stored at 4 °C. The washing step immediately decreased the viability of *L. monocytogenes* by 1.2–1.6 log units, however during storage the control over the pathogen growth was minimum.

In subsequent studies, enterocin AS-48 was assessed for the decontamination of soybean sprouts. Combinations of enterocin AS-48 (25 μg/ml) and polyphosphoric acid, in a concentration range of 0.1 to 2.0%, significantly reduced or inhibited growth of the populations of *S. enterica*, *E. coli* O157:H7, *Shigella* spp., *Enterobacter aerogenes*, *Yersinia enterocolitica*, *A. hydrophila* and *P. fluorescens* in sprout samples stored at 6 °C and 15 °C (Molinos et al., 2008a). The bacteriocin washing was also tested to inhibit *Bacillus* spp. on alfalfa, soybeans sprouts and green asparagus. The treatment with enterocin reduced viable cell counts of *Bacillus cereus* and *Bacillus weihenstephanensis* by 1.0–1.5 and by 1.5–2.4 log units, respectively. The bacteriocin was effective in reducing the remaining viable population below detection levels during storage at 6 ºC. Application of washing treatments containing enterocin AS-48 in combination with several other antimicrobials and sanitizers was also tested. The combinations of AS-48 and sodium hypochlorite, peracetic acid or hexadecylpyridinium chloride provided the best results. After application of the combined treatments, *B. cereus* and *B. weihenstephanensis* were not detected or remained at very low concentrations in the sprouts treated, along the storage period at 15 °C (Molinos et al., 2008b).

Bacteriocin RUC9, produced by a wild strain of *Lac. lactis,* was tested as a washing solution of minimally processed Iceberg lettuce to control *L. monocytogenes* during storage at 4 °C. The treatment resulted in a reduction of 2.7 log units of *L. monocytogenes* counts after 7 days, however it was not effective in removing completely the pathogen from the produce (Randazzo et al., 2009).

Anacarso et al. (2011) investigated the effect of adding Enterocin 416K1, alone or in combination with chitosan, to zucchini, corn, radishes, mixed salad and carrots on the *L. monocytogenes* population. When both antibacterial substances were used, the minimal

reduction achieved on *Listeria* population was at least 2 log units. In addition, the *L. monocytogenes* reduction achieved with the enterocin 416k1 alone was almost comparable with the antibacterial activity observed for the combination of bacteriocin and chitosan.

In the study by Oliveira et al. (2015), where nisin washing was evaluated, a 1.8 log unit reduction on the *L. monocytogenes* numbers present on fresh cut lettuce during storage at 10 ºC was observed.

Recently, Ramos et al. (Submitted to Food Microbiology) revealed that a washing step using a pediocin DT016 based solution prevented the proliferation of *L. monocytogenes* on fresh lettuce, rocket salad, parsley and spinach. Moreover, when comparing with a commercial available hypochlorite sodium solution, the pediocin solution resulted on lower pathogen loads, by at least 2.7 log unit on all the fresh vegetables.

The bacteriocins from Gram (-) bacteria can be useful in the control of enteric pathogens. Numerous Gram (-) species produce bacteriocins, but those produced by *E. coli* strains (or colicins) are the best studied (Gálvez et al., 2011). Semi-crude colicin Hu194, produced by *E. coli* strain Hu194, was applied in alfalfa seeds contaminated with *E. coli* O157:H7 strains. The bacteriocin treatment successfully reduced the pathogen viable counts (≈ 5 log CFU/g reduction) from the alfalfa seeds (Nandiwada et al., 2004).

Table 6. Bacteriocin applications to fresh and minimally processed vegetables

Biopreservation agent	Application	Target microorganism	Food	Reference
Mundticin	Washing solution Coating	*Listeria monocytogenes*	Mung bean sprouts	(Bennik et al., 1999)
Colicin Hu194	Additive Coating	*Escherichia coli* O157:H7	Alfalfa seeds	(Nandiwada et al., 2004)
Nisin Pediocin alone, and in combination with other antimicrobial compounds	Washing solution	*Listeria monocytogenes*	Fresh cut cabbage Broccoli Mung bean sprouts	(Bari et al., 2005)
Bacteriocin LABB Bacteriocin LABP Nisin	Washing solution	*Listeria monocytogenes* *Staphylococcus aureus*	Vegetable model system	(Jamuna et al., 2005)
Enterocin AS-48, and in combination with other antimicrobial compounds	Washing solution	*Listeria monocytogenes*	Alfalfa sprouts Soybean sprouts Green asparagus	(Molinos et al., 2005)
Nisin Coagulin Nisin and coagulin	Washing solution	*Listeria monocytogenes*	Fresh cut lettuce	(Allende et al., 2007)

Table 6 (continued). Bacteriocin applications to fresh and minimally processed vegetables

Biopreservation agent	Application	Target microorganism	Food	Reference
Enterocin AS-48 (25 µg/ml) with polyphosphoric acid (0.1 to 2.0%)	Washing solution	*Salmonella enterica* *Escherichia coli* O157:H7 *Shigella* spp. *Enterobacter aerogenes* *Yersinia enterocolitica* *Aeromonas hydrophila* *Pseudomonas fluorescens*	Soybean sprouts	(Molinos et al., 2008a)
Enterocin AS-48, and in combination with other antimicrobial compounds	Washing solution	*Bacillus cereus* *Bacillus weihenstephanensis*	Alfalfa sprouts Soybean sprouts Green asparagus	(Molinos et al., 2008b)
Bacteriocin RUC9	Washing solution	*Listeria monocytogenes*	Minimally processed Iceberg lettuce	(Randazzo et al., 2009)
Enterocin 416K1, and in combination with chitosan	Additive	*Listeria monocytogenes*	Zucchini Corn Radishes Mixed salad Carrots	(Anacarso et al., 2011)
Nisin	Washing solution	*Listeria monocytogenes*	Fresh cut lettuce	(Oliveira et al., 2015)

Table 6 (continued). Bacteriocin applications to fresh and minimally processed vegetables

Biopreservation agent	Application	Target microorganism	Food	Reference
Pediocin DT016	Washing solution	*Listeria monocytogenes*	Iceberg lettuce Rocket salad Spinach Parsley	(Ramos et al., Submitted to Food Microbiology)

2.5. Bacteriophages

Bacteriophages or phages are the microorganisms most abundant in Earth (10^{31} particles) and are present in numerous foods. Bacteriophages are viruses that specifically infect bacterial cells, being harmless to humans, animals and plants (García et al., 2010). They are considered safe and represent a potential natural intervention to reduce human pathogens from vegetables (Anany et al., 2015, Żaczek et al., 2015).

Bacteriophages have an average size of 20 to 200 nm and possess two main components: genetic material in the form of DNA or RNA as a core, and a surrounding protein or lipoprotein shell (capsid). The core nucleic is connected with a tail that interacts with various bacterial surface receptors via the tip of the tail fibers. This interaction shows an affinity that is specific to certain group of bacteria or even to a particular strain (Anany et al., 2015, Hagens and Loessner, 2010).

The phages are classified into 13 families based on their shape, size and type of nucleic acid, and presence/absence of envelope or lipids in their structure. Most of them are tailed bacteriophage, which accounts for 96 % of all phages present on earth, belonging to the order *Caudovirales.* According to the morphological features of the tail, they are classified into three families: the *Myoviridae* (long contractile tail), the *Siphoviridae* (long non contractile tail) and the *Podoviridae* (short non contractile tail) (Deresinski, 2009, García et al., 2010).

As bacteria's parasites, bacteriophages start the infection with adsorption to the suitable host cell, followed by injection of their genetic material into the bacterial cytoplasm (Sharma and Sharma, 2012). The bacteriophages can be divided in two groups, lytic (virulent) or lysogenic (temperate) bacteriophages, depending on their life cycle.

For lytic phages, the bacteriophage genes are transcribed by the host cell machinery, the virion particles are assembled and the lysis of the peptidoglycan layer releases virion particles from the cell, which allow the particles to infect other bacterial hosts (Anany et al., 2015, Sharma and Sharma, 2012). On the other hand, for temperate bacteriophages the phage genome remains in a repressed state in the host genome and it is replicated as

part of the bacterial chromosome until lytic cycle is induced. Therefore, temperate phages are not suitable for biocontrol (Anany et al., 2015).

Bacteriophages lytic action is specific to groups or species of bacteria. This allows their use to target pathogenic bacteria, without substantially changing the microbial ecology or microbiota of the produce commodity. This specificity at strain level can be a limitation for application of bacteriophages. Nevertheless, several studies have shown the efficacy of mixtures containing different bacteriophages and broad host range bacteriophages to attack a high number of bacterial strains, including the most virulent strains found in foods (Gálvez et al., 2014, Hagens and Loessner, 2010). Bacteriophages selected to be used as biopreservation agents may have the desirable properties listed in Table 7.

Table 7. Desirable properties of bacteriophages to be applied as biopreservation agent to fresh foods

Microorganism
Broad range
No interference with microbiota
Strictly lytic (virulent)
Propagated on pathogenic and non-pathogenic host
Increase in numbers through lytic infection
Lack of transduction of non-viral (i.e., bacterial) DNA
Effective at low concentrations
Health
Non toxic
GRAS status
Absence of any genes encoding pathogenicity associated or potentially allergenic proteins
No adverse effects
Complete genome sequences known
Product
pH and Temperature tolerant
Stable over application and storage conditions
No negative organoleptic effects
Reduce the use of chemical preservatives
Potential to scale up for commercial production
Biological characteristics can be inferred from simple experiments and bioinformatics analysis
Less severe than heat treatments
Compatible with other control systems, and therefore can be applied together (hurdle technologies)

2.5.1. Applications of bacteriophages to fresh and minimally processed vegetables

There are several bacteriophage preparations commercially available for food safety applications, such as ListShield™ and EcoShield™ (Intralytix, Inc., USA), Agriphage™

(Omnilytics, Inc., USA), and Listex™ P100 and Salmonelex™ (Micreos Food Safety, The Netherlands) (Boyacioglu et al., 2013, Mahony et al., 2011). The approval of using phage preparations in food products by U.S. Food and Drug Administration (USFDA) provided the impetus for further investigation into their applications. A summary of bacteriophages applications in fresh and minimally processed vegetables is presented in Table 8.

Pao et al. (2004) isolated two bacteriophages capable of lysing *Salmonella,* phage A and phage B, from sewage water and assessed their potential to control *Salmonella* spp. on sprouting seeds. They found that bacteriophages application resulted in a 1.5 log suppression of *Salmonella* growth in the soaking water of broccoli seeds. When only phage A was applied, a 1.4 log suppression of the pathogen growth was achieved on mustard seeds.

Abuladze et al. (2008) showed that bacteriophages may be useful for reducing contamination of various vegetables by *E. coli* O157:H7. In particular, ECP-100 (now EcoShield™ (Intralytix, Inc., USA)), a bacteriophage cocktail containing three *Myoviridae* phages lytic for *E. coli* O157:H7, was applied to decontaminate tomato, spinach and broccoli. Treatments with the ECP-100 preparations resulted in *E. coli* reductions ranging from 94% (tomato) to 100% (spinach).

In a broad set of experiments, a virulent broad host range phages revealed to be very effective for specific biocontrol of *L. monocytogenes* in ready to eat foods. Bacteriophages A511 and P100 reduced the pathogen load by 5 log units in sliced cabbage and lettuce leaves (Guenther et al., 2009).

Kocharunchitt et al. (2009) suggested the existence of a temporary, acquired, non-specific phage resistance phenomenon by bacteria. Briefly, two *Salmonella* bacteriophages (SSP5 and SSP6) were evaluated for their potential to control *Salmonella* Oranienburg on alfalfa seeds. Addition of phage SSP6 to alfalfa seeds, previously contaminated with the pathogen, caused approximately 1 log unit reduction of viable *Salmonella*. However, thereafter the phage had no inhibitory effect on the pathogen growth.

ECP-100 was an effective treatment to reduce *E. coli* 0157:H7 on fresh cut lettuce stored at refrigeration temperature (4 ºC) (Sharma et al., 2009).

As referred previously, using a combination of bacteriophages with *Ent. asburiae* JX1, the levels of *Salmonella* spp. associated with mung bean sprouts were only detected by enrichment (Ye et al., 2010).

In experiments by Viazis et al. (2011), organic baby spinach and baby romaine lettuce leaves artificially contaminated with *E. coli* O157:H7 were treated with BEC8, a mixture of eight lytic bacteriophages, at different multiplicity of infection levels (MOI 1, 10 and 100) and under various conditions. The treatment reduced the number of *E. coli* O157:H7 cells in the produce and it was observed that higher MOI, temperature and incubation period resulted in greater bacterial inactivation. The authors also demonstrated that phage treatment combined with the essential oil trans-cinnamaldehyde was more effective than the phages alone, and the most environment friendly way to reduce bacterial contamination from food.

Several studies assessed the effect of EcoShield™ (Intralytix, Inc., USA) on vegetables artificially contaminated with *E. coli* O157:H7. Carter et al. (2012) studied the capacity of this bacteriophage cocktail in lower concentrations to inhibit the pathogen from lettuce. It was concluded that the phage treatment was effective in reducing the bacterial count by 1–2 logs on lettuce and in maintaining the pathogen levels during the storage period at 4 ºC. However, the reduction in the phage concentration slightly reduced its efficacy. Boyacioglu et al. (2013) and Ferguson et al. (2013) considered the application of this preparation in fresh cut leafy greens. Boyacioglu et al. (2013) contaminated fresh spinach and romaine lettuce with *E. coli* O157:H7 and sprayed the fresh cut leaves with an EcoShield™ solution. In another experiment Ferguson et al. (2013) studied the potential effect of EcoShield™ in preventing cross contamination of Iceberg lettuce, by treatment of the produce with bacteriophage solution and later contamination with *E. coli* O157:H7. In both cases, phage application significantly reduced the pathogen population in produce. Boyacioglu et al. (2013) observed the effect of the phage treatment as early as 30 min after EcoShield™ spraying, and this was maintained over the storage period at 4 ºC. In studies by Ferguson et al. (2013) preventive phage application did not immediately cause reduction in *E. coli* cells, but was most successful after several days of storage at 4 °C. The presented experiments indicate that EcoShield™ has the potential to inhibit

growth of *E. coli* O157:H7 in ready to eat vegetables and can be used as a biocontrol tool in the food industry.

Spricigo et al. (2013) reported the effectiveness of a bacteriophage cocktail (UAB_Phi 20, UAB_Phi78, and UAB_Phi87) in reducing *Salmonella enterica* serovar Typhimurium and *S. enterica* serovar Enteritidis loads from lettuce. A significant bacterial reduction (2.2 log CFU/g) was obtained in the lettuce samples dipped in a solution containing the bacteriophage cocktail for 60 min at room temperature.

Washings with lytic bacteriophage cocktails, levulinic acid and a combination of both treatments were investigated for their effectiveness against the foodborne pathogens: *E. coli* O157:H7, *Shigella* spp. and *Salmonella* on broccoli. The combination of both treatments was effective in reducing the pathogens from produce and was not influenced negatively by the presence of high loads of organic matter (Magnone et al., 2013).

Perera et al. (2015) observed that ListShield™ treatment of lettuce reduced *L. monocytogenes* cells by 1.1 log units.

In the experiments by Oliveira et al. (2015) it was additionally demonstrated that Listex™ P100 and Salmonelex™ treatments were not efficient in reducing, respectively, *L. monocytogenes* and *Salmonella* spp. populations from fresh cut lettuce. This highlighted that effective biocontrol strategies may need to be combined with other technologies.

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Table 8. Bacteriophages applications to fresh and minimally processed vegetables

Biopreservation agent	**Application**	**Target microorganism**	**Food**	**Reference**
Phage A and phage B	Additive	*Salmonella* spp.	Broccoli seeds Mustard seed	(Pao et al., 2004)
ECP-100 (cocktail of three lytic phages ECML-4, ECML-117, ECML-134)	Spraying solution	*Escherichia coli* O157:H7	Broccoli Spinach	(Abuladze et al., 2008)
Bacteriophages A511 and P100	Additive	*Listeria monocytogenes*	Fresh cut cabbage Fresh cut lettuce	(Guenther et al., 2009)
Bacteriophage SSP 5 Bacteriophage SSP6	Washing solution	*Salmonella* Oranienburg	Alfalfa seeds	(Kocharunchitt et al., 2009)
ECP-100 (cocktail of 3 lytic phages)	Spraying solution	*Escherichia coli* 0157:H7	Fresh cut lettuce	(Sharma et al., 2009)
BEC8 (mixture of eight lytic bacteriophages), and in combination with	Additive	*Escherichia coli* 0157:H7	Baby spinach Baby romaine lettuce leaves	(Viazis et al., 2011)
EcoShield™ (cocktail of three lytic phages)	Spraying solution	*Escherichia coli* 0157:H7	Lettuce	(Carter et al., 2012)

Table 8 (continued). Bacteriophages applications to fresh and minimally processed vegetables

Biopreservation agent	Application	Target microorganism	Food	Reference
EcoShield™ (cocktail of three lytic phages)	Spraying solution	*Escherichia coli* O157:H7	Fresh spinach Romaine lettuce	(Boyacioglu et al., 2013)
EcoShield™ (cocktail of three lytic phages)	Additive	*Escherichia coli* O157:H7	Iceberg lettuce leaves	(Ferguson et al., 2013)
Bacteriophage cocktail, (UAB_Phi 20, UAB_Phi78, and UAB_Phi87)	Washing solution	*Salmonella enterica* serovar Typhimurium *Salmonella enterica* serovar Enteritidis	Romaine Lettuce	(Spricigo et al., 2013)
Bacteriophage cocktail , and in combination with levulinic acid	Washing solution	*Escherichia coli* O157:H7 *Shigella* spp. *Salmonella* spp.	Broccoli	(Magnone et al., 2013)
Listex P100, Salmonelex	Washing solution	*Listeria monocytogenes* *Salmonella* spp.	Fresh cut lettuce	(Oliveira et al., 2015)

2.6. Conclusions

Application of live cultures to vegetables, calls for innovative multidisciplinary inputs from various fields of food science. Protective culture applications offer advantages, such as shelf-life extension and food safety improvements. This review highlights the importance of the careful isolation and selection of biopreservation strain(s). Protective cultures efficacy is affected by the inoculation level, the microbiota, the physic-chemical and structure of the products, and the storage conditions. In the future, combined protective culture preparations could be possible.

Bacteriocins, applied directly or as a washing treatment, have shown to be an effective approach to improve microbial safety and reduce the chemical treatment in minimally processed vegetable processing. However, bacteriocins are affected by numerous factors, e.g. they may be efficient only in a narrow pH range, which excludes their utilization in many food products. Thus, a single bacteriocin-based technique is specific to a single food matrix, and its application on different matrices needs to be tested. The combination of bacteriocins with other antimicrobial compounds, and with other preservative techniques, can overcome this limitation and further improve the safety of the products.

In the last few years, several authors have assessed the potential of using bacteriophages in biocontrol of human foodborne pathogens. One important advantage of their use, is that they may enable targeted elimination of a specific pathogenic bacteria in foods without affecting the microbiota of the foods.

Bacteriophages applications in the vegetable processing needs to be further studied. For example, bacteriophages can act synergistically with antagonistic bacteria against selected human pathogenic bacteria, this is an interesting approach that has very seldom been exploited. The lytic enzymes produced by bacteriophages could also be exploited as antimicrobial agents, minimizing the impact of phage specificity. The combination of different biopreservation agents represents a promising, chemical-free approach for controlling the growth of foodborne pathogens in vegetables.

Commercial applications of biopreservation methods require a wider availability of protective cultures, bacteriocins and bacteriophages with limited or known sensory

effects. In addition, more information about their health and toxicological effects and safety is required before legal acceptance. Therefore, additional studies using biopreservation approaches, or combined methods, to extend and enhance the safety of this kind of products are crucial.

2.7. Acknowledgements

This work was supported by National Funds from FCT - Fundação para a Ciência e a Tecnologia through project UID/Multi/50016/2013.
Financial support for authors Ramos B. and Brandão T.R.S. was provided by FCT and Fundo Social Europeu (FSE) through fellowships SFRH/BD/42169/2007 and SFRH/BPD/101179/2014, respectively.

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Part II – Natural Approaches

Chapter 3. Balsamic Vinegar from Modena: An easy and accessible approach to reduce *Listeria monocytogenes* from lettuce

Chapter 4. Antilisterial active compound from lactic acid bacteria present on Iceberg lettuce.

Chapter 5. Biopreservation approaches to reduce *Listeria monocytogenes* in fresh vegetables

Chapter 6. General conclusions and Suggestion for further work

Chapter 3

Balsamic Vinegar from Modena: An easy and accessible approach to reduce Listeria monocytogenes from lettuce

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Food Control (2014), 42**,** 38-42.

3.1. Abstract

The microbiological safety of fresh produce is a significant concern of consumers and industry. After applying at an inoculated level (6-7 log CFU/mL) of *Listeria monocytogenes* on Iceberg lettuce, the antilisterial properties of balsamic vinegar from Modena, white wine vinegar and acetic acid solutions were investigated.

Different proportions of the vinegars, acetic acid (58.7 g/l), and distilled water, were evaluated to determine the role of those solutions at the stage of washing Iceberg lettuce to remove *L. monocytogenes*. The maximum observed log reduction of *L. monocytogenes* was 2.15 ± 0.04 for balsamic vinegar (50 % (v/v)), 1.18 ± 0.06 for white wine vinegar ((50 % (v/v)) and 1.13 ± 0.06 for acetic acid ((50 % (v/v)). Washing with water only reduces 0.05 ± 0.04 log CFU/mL of *L. monocytogenes* numbers.

Listeria reductions observed for balsamic vinegar are similar or higher than those of chlorine-based sanitizers evaluated in other studies with lettuce. In the case of balsamic vinegar solutions, *Listeria* inhibition followed a linear reduction according to the model: Log (N/N0) = - 4.09 x balsamic vinegar proportion % (v/v) – 0.13; $R^2 = 0.95$. Balsamic vinegar washings may be a promising method for reducing other foodborne pathogens present in produce or other foods, at home and retail environments.

Keywords: *Listeria monocytogenes*, Iceberg lettuce, balsamic vinegar from Modena, acetic acid, antilisterial activity

3.2. Introduction

The incidence of foodborne infections caused by bacterial pathogens continues to be a problem in industrialized nations and developing countries (Chang and Fang, 2007, Ramos-Villarroel et al., 2012). Bacteria most frequently linked to food outbreaks are

Salmonella spp., *Escherichia coli, Listeria monocytogenes* and *Shigella* spp. (Rico et al., 2007, Senorans et al., 2003, Warriner, 2005).

Gastrointestinal disease caused by *L. monocytogenes* is rare compared to other agents of foodborne illness, but invasive listeriosis can be very severe and has a high fatality rate (Little et al., 2007, Miller et al., 2009b, Nastou et al., 2012). This pathogen is considered ubiquitous in nature environment and produce is likely to be contaminated (Sant'Ana et al., 2012) .

Several studies have reported that the occurrence of *L. monocytogenes* in ready-to-eat vegetables in several parts of the world may be as high as 25 % (Cordano and Jacquet, 2009, Crepet et al., 2007, Sant'Ana et al., 2012). This is of special concern because this kind of food is likely to be consumed raw, relying only on cold storage to maintain their safety, but *Listeria* has the ability to survive and multiply at refrigeration temperatures (Luber et al., 2011, Miller et al., 2011).

Vegetable consumption has grown over the last two decades, especially lettuce that on average is eaten by a third of the population once a week (Doménech et al., 2013). Raw vegetables have been identified as a vehicle of transmission of foodborne outbreaks and play an important role in listeriosis epidemiology. Improper temperature control, poor cleanliness and inappropriate refrigerator management have been identified as critical factors in foodborne listeriosis (Luber et al., 2011, Ramos et al., 2013, Sant'Ana et al., 2012).

Proper food handling at home can maintain the hazard at a safe level and even reduce it. Thus, it is important to develop strategies to control *L. monocytogenes* in the home environment (Doménech et al., 2013, Shen et al., 2009).

Retail environments also play a role in the contamination of foods and/or amplification of *L. monocytogenes*, however linking a specific retail environment to an outbreak of infection is difficult to prove (Varma et al., 2007). Interventions directed at home and retail environments may be an important way to reduce sporadic disease, which represents the greatest burden of *L. monocytogenes* infection (Varma et al., 2007).

In the home or restaurant, fewer options are available for effective washing of vegetables than in a modern processing plant. There are various chemical compounds available in

these environments that can be useful for sanitizing fresh produce, particularly vinegar which contains acetic acid (Shen et al., 2009, Yang et al., 2009).

Vinegar and vinegar-based solutions are commonly used as dressings for salads and appetizers and have been studied with favourable results for their efficacy in removing pathogens from fresh fruits and vegetables (Chang and Fang, 2007, Sengun and Karapinar, 2004, Shen et al., 2009, Vijayakumar and Wolf-Hall, 2002). In addition, consumers are increasingly avoiding consumption of foods treated with preservatives of chemical origin and so vinegar solutions can be an appealing natural alternative (Sengun and Karapinar, 2004).

The purpose of this study was to determine the antimicrobial activity of vinegar solutions on *L. monocytogenes* inoculated onto lettuce. With this aim in mind, inactivation of this microorganism with different washing solutions was determined

3.3. Materials and Methods

3.3.1. Preparation of Listeria monocytogenes inoculum

3.3.1.1. Cultures

A three-strain composite of *L. monocytogenes* was used in this study. These strains were 1334 serotype 1/2c, 1336 serotype 1/2b and 1092 serotype 4b (Escola Superior de Biotecnologia, UCP).

Listeria monocytogenes strains were grown independently for 24h at 37 ºC in 50 mL Tryptic Soy Broth (Lab M, Lancashire, UK) with 0,6 % yeast extract -TSBYE (Lab M).

3.3.1.2. Preparation of cultures

The second subculture of each strain was incubated at 37 °C for 24 h to yield stationary phase cultures. This cell growth phase was chosen due to its higher stress resistance than exponential phase cells (Miller et al., 2009a).

The three cultures were mixed together in the same proportion, and washed twice by centrifuging (5000 rpm, 5 min, 4 ºC) with sterile distilled water. The cell pellets were resuspended in distilled water so that final cell numbers in the suspension were approximately 6-7 log CFU/mL.

3.3.2. Procedure for inoculating lettuce

Iceberg lettuce (*Lactuca sativa capitata)* was purchased from a local supermarket, outer layers were removed, and the leaves were portioned by hand. All lettuces were kept at 4–5 ºC between the time of purchase and initiation of experiments, and were then used immediately.

Lettuce was dipped into *L. monocytogenes* culture suspension for 15 min and placed on sterile paper for removing excess liquid at room temperature (20 ºC) and transferred to sterile bags. To facilitate the attachment of bacteria, samples were stored for 24 h at 4-5 ºC before they were treated with the solutions.

3.3.3. Preparation of treatment solutions

Preliminary studies, using the agar diffusion method, were made to establish the antilisterial activity of vinegars commercially available: traditional balsamic vinegar from Modena (details further in text; referred to only as balsamic vinegar), rice, fruit, white and red wine and cider vinegars. Balsamic vinegar showed the best antilisterial activity and for that reason was chosen for future analyses (data not shown). Wine vinegar (later

referred only as white vinegar) was also selected due to its common use and presence in households.

Vinegars and acetic acid solutions, for the dipping treatment, were prepared immediately before use. The control solution was done with sterile distilled water alone.

Vinegar solutions were made by dilution in distilled water, to achieve the following vinegar proportions: 15, 20, 37 and 50 % (v/v).

Acetic acid concentration of vinegars, determined according to NP 3264:1989, was 58.7 and 61.5g acetic acid/L, for balsamic and wine vinegar, respectively.

A solution with a concentration of 58.7g acetic acid/L was prepared from glacial acetic acid (Panreac, Barcelona, Spain) and diluted in distilled water in the same proportions as the previous vinegar solutions.

The pH value of the solutions was measured using a pH meter (GLP 22, Crison Instruments, Spain) and the mean values are presented in Table1.

Table 1. pH values for the different tested solutions

Compound	Proportion % (v/v)	pH
Balsamic Vinegar from Modena	15	3.38
	20	3.35
	37	3.29
	50	3.26
White Vinegar	15	3.05
	20	3.00
	37	2.93
	50	2.89
CH_3COOH 58.7 g/L	15	2.92
	20	2.88
	37	2.73
	50	2.65

3.3.4. Washing treatments

Inoculated lettuce, 500 g approximately, was added to 1 L of the treatment solutions (at 20 ºC), sufficient to cover all pieces, and left for 15 min at room temperature. After the samples were removed, they were placed on sterile absorbent paper to allow removal of the excess liquid.
All experiments were made in triplicate

3.3.5. Enumeration of L. monocytogenes

Listeria enumeration was done before and after the washing procedures. A 25 g sample of lettuce was aseptically transferred to 225 mL of buffered peptone water (Lab M, Lancashire, UK) in a stomacher bag and homogenized in a Stomacher (Lab-Blender 400, Seward Medical, London, UK) for 90 s. Each sample was serially diluted and plated in duplicate onto Palcam agar (Merck, Darmstadt, Germany) plus selective supplement (Merck, Darmstadt, Germany). Typical colonies were counted after incubation at 37 ºC for 48 h to determine the survival of *L. monocytogenes*.
Mean values of bacterial counts (CFU/g), from duplicate plate samples were converted to log numbers for each combination.

3.3.6. Data analysis

In terms of microbial loads, the treatment effects were assessed by calculating the reduction of microbial content in relation to fresh untreated samples, expressed in terms of log-cycles (i.e. log (N/N_0), where N_0 is the sample initial microbial load and N is the microbial load after treatment). Microsoft® Excel 2010 (Microsoft Corporation, Washington USA) was used for all calculations analysis.
A two-way ANOVA was used to assess the influence of treatment solutions and corresponding concentrations on *Listeria* inactivation. Multiple comparisons on mean

values of *Listeria* enumerations were evaluated by Tukey's post-hoc test using SPSS statistics 20 (IBM, New York, USA). The level of significance for all tests was 0.05.

3.4. Results and Discussion

Viable *L. monocytogenes* reductions obtained after washing were relative to populations on inoculated lettuce (positive control). The inoculation level used in the experiment was higher than natural contamination to allow valid observation of bacterial reductions after washing with different solutions.

This study revealed that the usual method, for home and retail environments, of water dipping lettuce with water is not effective in removing *Listeria* from lettuce. Water dipping only decreased 0.05 ±- 0.04 log of *L. monocytogenes* inoculated on lettuce. The various proportions of balsamic vinegar washings resulted in different bacteria reductions, and the increment of vinegar solution % was followed with increasing reductions of bacterial numbers ($p<0.05$). In fact, *Listeria* destruction followed a linear reduction according to the model: Log (N/N_0) = - 4.09 balsamic vinegar proportion % (v/v) – 0.13; $R^2 = 0.95$. Data and model fit are shown in Figure 2.

Populations of *L. monocytogenes* were reduced (0.86 ± 0.02 log CFU/g) significantly ($p<0.05$) when the samples were dipped in 15 % (v/v) of acetic acid solution. However, increasing the proportion of acetic acid from 20 to 50% (v/v) did not result in any further decrease (1.13 log CFU/g; $p>0.05$). For increased proportions of white vinegar, different reductions were obtained ($p<0.05$) with the exception of 37 % (v/v).

Overall acetic acid and white vinegar solutions showed similar efficiency on removing *L. monocytogenes* from lettuce ($p>0.05$).

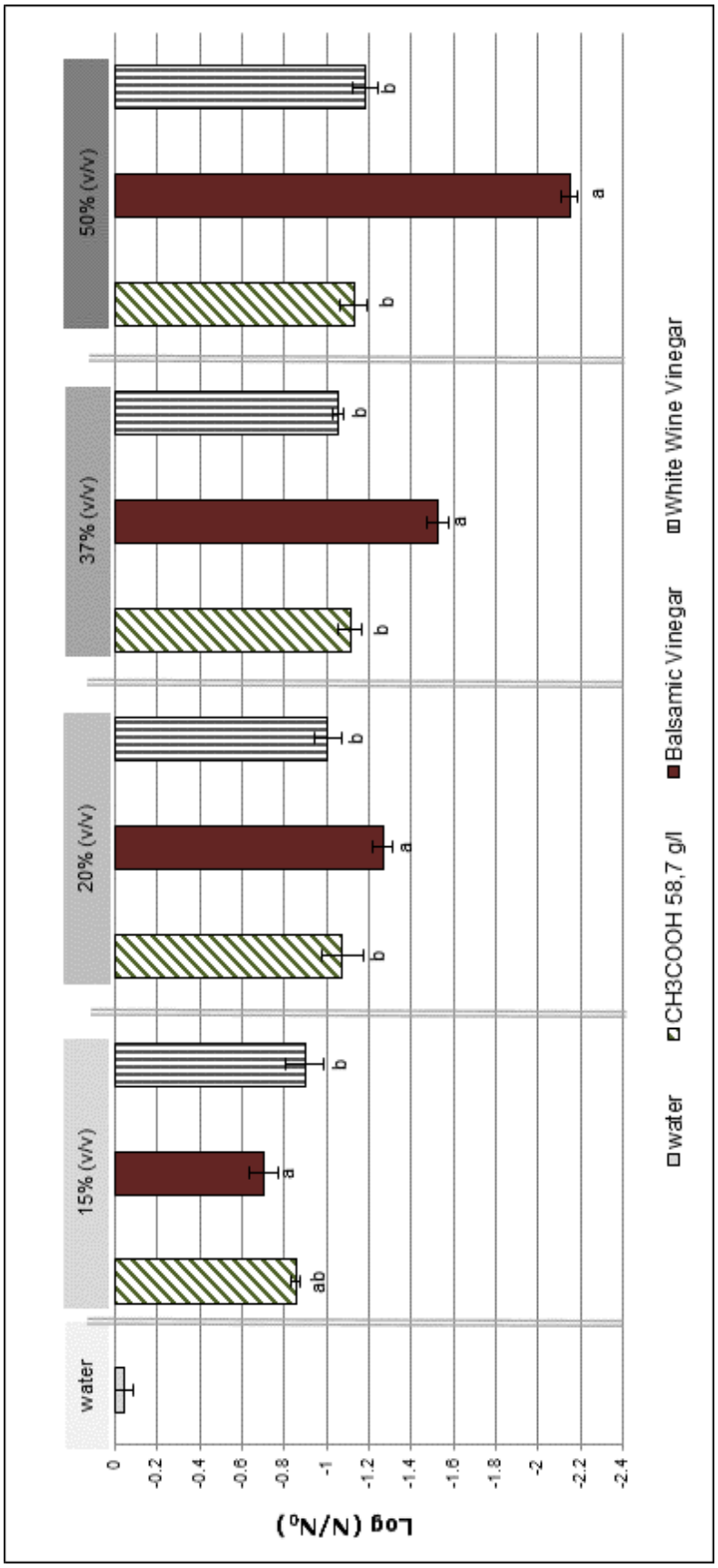

Figure 1. Antilisterial activity of the different washing solutions and proportions, shown in terms of Log (N/N0). For a given washing proportion, values with different letters differ significantly ($p<0.05$).

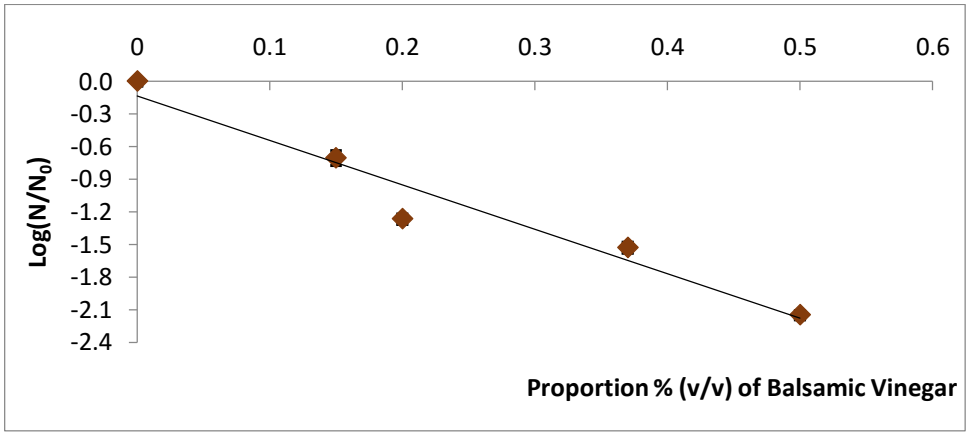

Figure 2. Relation between the *Listeria* reduction and balsamic vinegar proportion. The line represents linear model fit: Log (N/N_0) = - 4.09 balsamic vinegar proportion % (v/v) – 0.13, $R^2 = 0.95$.

According to the present results, dipping lettuce in 1.0-2.9 % g/L acetic acid resulted in 1.08-1.13 log CFU/g reduction. Akbas and Ölmez (2007) reported that populations of *L. monocytogenes* on Iceberg lettuce were reduced (0.9 log CFU/g).

In a study by Samara and Koutsoumanis (2009), dipping lettuce in 0.5 % and 1.0 % acetic acid reduced *L. monocytogenes* by less than 1 log CFU/cm^2. Our data is in general agreement with most other studies reporting that acetic acid concentrations of up to ~1.0 % are unlikely to reduce *L. monocytogenes* populations by more than about 1 log CFU/g (Nastou et al., 2012, Samara and Koutsoumanis, 2009, Zhang and Farber, 1996). Other studies with organic acids (0.25 g/ 100 g citric acid plus 0.50 g/100 g ascorbic acid) reported similar listeria reductions to those obtained with white wine vinegar and acetic acid solutions (Ölmez and Temur, 2010).

Efficacy of balsamic vinegar at 50 % (v/v) to decontaminate *L. monocytogenes* from lettuce surfaces was similar or higher to those of chlorine-based sanitizers evaluated in other studies (Akbas and Olmez, 2007, Akbas and Ölmez, 2007, Behrsing et al., 2000, Doménech et al., 2013, Kilonzo-Nthenge et al., 2006, Park et al., 2011, Zhang and Farber, 1996). Ölmez and Temur (2010) reported similar *Listeria* reductions, 2.3 and 2.2 log

CFU/g with chlorine (100 mg/L) and ozone (2mg/L) respectively. Park et al. (2011) reported higher antilisterial activity linked to malic, citric and lactic acid, however in that study they only allowed bacterial attachment for 3h. This increase in the efficacy of sanitizing treatments may be explained by the initiation of biofilm formation whereas there is an increase in the development of cell aggregates after 24 h of incubation (Ells and Truelstrup Hansen, 2006, Koseki et al., 2001). The strength of attachment is a main factor affecting the efficacy of sanitizing treatments (Ölmez & Temur, 2010).

The higher antimicrobial activity of balsamic vinegar solutions was not only due to hydrogen ion effect, since the pH of the balsamic vinegar solutions ranged from 3.26-3.38 while the white vinegar and the acetic acid solutions had pH values of 2.89-3.05 and 2.65-2.92, respectively. Also the acid present in all the solutions was mostly present in undissociated form (pH < pKa (CH_3COOH)).

The stronger bactericidal effect of balsamic vinegar may be also related to the presence of compounds with antimicrobial properties resulting from the fermentation of grape juice and from grape juice itself. It is known that grapes contain a number of phenolic compounds that exhibit antilisterial activity, particularly polymeric phenolic compounds: resveratrol, vanillic acid, caffeic acid, gallic acid and flavonoids (rutin and quercetin) (Baydar et al., 2004, Oliveira et al., 2013, Rhodes et al., 2006, Rodríguez Vaquero et al., 2007). In fact Plessi et al. (2006) found three of these antilisterial compounds, vanillic acid, gallic acid and caffeic acid in traditional balsamic vinegar from Modena.

3.5. Conclusions

All tested solutions showed higher bactericidal effects against the *L. monocytogenes* strains than water, although the balsamic vinegar activity was clearly higher.

Balsamic vinegar showed similar and even better effectiveness than chlorine-based sanitizers on removing *L. monocytogenes* from lettuce surface, even though the time of storage of inoculated lettuce allowed the formation of biofilms. The presence of phenolic

compounds naturally presented in grape and grape juices may be responsible for its high antilisterial activity.

Balsamic vinegar washing seems to be a promising method to reduce *L. monocytogenes* present in produce at home and retail environments. Good results at home or retail may be achieved simply adding a cup of vinegar (240 mL) to a cup of water (240 mL) and dip the vegetables for 15 min.

Balsamic vinegar may be a promising effective solution to inhibit other food pathogens present on produce surface or other foods. There is a lack of studies with these vinegars and it is an important resource for households and food establishments due to is availability and organic nature.

3.6. Acknowledgements

This work was supported by National Funds from FCT – Fundação para a Ciência e a Tecnologia through project PEst-OE/EQB/LA0016/2011.

Financial support for authors Ramos B., Miller F.A. and Brandão T.R.S. was provided by FCT and Fundo Social Europeu (FSE) through fellowships SFRH/BD/42169/2007, SFRH / BPD / 65041 / 2009 and SFRH / BPD / 41419 / 2007, respectively.

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Chapter 4

Antilisterial active compound from lactic acid bacteria present on Iceberg lettuce

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Acta Alimentaria (2016), 45 (3), 417–427.

4.1. Abstract

Pediococcus pentosaceus DT016, a bacteriocin producing strain, was isolated from fresh lettuce. A protein with antilisterial activity (bacDT016), between 11 to 17 kDa, was identified and characterized as the bioactive substance from the LAB culture. Highest bacteriocin production was noted after 15 h of growth. Antibacterial activity was affected by some enzymes and detergents, as well as by temperatures equal or above 80 ºC. DT016 strain contains a 1110 bp DNA fragment with homology to pediocin AcH/PA-1.

Keywords: *P. pentosaceus* DT016, lettuce, bacteriocin, antilisterial activity, *L. monocytogenes*

4.2. Introduction

The demand for fresh and minimally processed vegetables has increased in the last decades (Trias et al., 2008). These foods may be consumed raw or minimally processed, and therefore can be a vehicle of several pathogens.

Listeria monocytogenes is ubiquitous in the agricultural environment. Its occurrence in ready-to-eat vegetables may be as high as 25%. In fact, vegetables have been implicated in outbreaks of listeriosis, suggesting that lettuce can have a high incidence of *L. monocytogenes* (Ramos et al., 2014, Ramos et al., 2013).

Dairy cows and dairy farm environments are reservoirs of this pathogen, where faecal shedding contributes to its environmental dispersal and contamination of milk, dairy products, and meat represents a risk to consumers (Haley et al., 2015).

Meat raw materials have often been related with outbreaks of human listeriosis. In effect, a study carried out in Italy from 2008 to 2014, detected *L. monocytogenes* with an

occurrence of 4.2 % in raw pork sausages and of 2.4 % in entrails lamb rolls (D'Ostuni et al., 2016).

Seafood, fish and fishery products are considered as the most frequent causes of a number of sporadic listeriosis cases. The contamination of these products with this pathogen is very likely through contaminated waters and environments, during transportation and in fish markets. *L. monocytogenes* was isolated from raw fish (6.9 %) and from open-air fish market environment (2.5 %), in Iran (Jamali et al., 2015).

An increasing number of consumers prefer foods, prepared without chemical preservatives to guarantee the microbiology safety. A promising alternative to the use of chemical additives is the use of lactic acid bacteria (LAB) and/or their natural metabolites to enhance food safety. Among these metabolites, bacteriocins are a heterogeneous group of peptides and proteins, able to kill or inhibit the growth of other bacteria (Jang et al., 2014, Jiang et al., 2012). Research on bioactive substances from LAB is important for their potential applications in the food industry and public health contribution (O'Bryan et al., 2015). Bacteriocinogenic LAB, originally isolated from vegetables, are probably the best candidates. Their antibacterial compounds may be used as a weapon to improve the safety of these products, while answering the need for effective biopreservation techniques (Wei et al., 2006).

In this study the antimicrobial activity of a LAB strain previously isolated from Iceberg lettuce was investigated.

4.3. Material and Methods

4.3.1. Antibacterial activity of lactic acid bacteria isolate

A lactic acid bacterium (DT016) isolated from Iceberg lettuce with activity against *L. monocytogenes*, *L. innocua* and *E. faecalis* was cultured in MRS broth (Lab M) and the antimicrobial activity and its nature were assessed according to Tomé et al. (2006). *L. innocua* 2030c*, L. monocytogenes* 1334 (1/2c), and *L. monocytogenes* 1336 (1/2b) were

used as target organisms; *Pediococcus acidilactici* HA-6111-2, a pediocin PA-1 producer (Albano et al., 2007) with activity against *L. monocytogenes,* was used as control.

4.3.2. Identification of bacteriocin-producing strain

Identification of the bacteriocin-producing strain was performed by PCR amplification of 16S rRNA gene as described by Pinto et al. (2009).

4.3.3. Bacteriocin production

An overnight culture of *P. pentosaceus* DT016 was inoculated in MRS broth (1% v/v) and incubated at 37 °C, without shaking. Changes in pH and optical density (600 nm) were recorded every hour, bacteriocin activity (arbitrary units per mL- AU ml^{-1}) and viable counts were calculated every 3 h, according to van Reenen et al. (1998). All experiments were made in triplicate and each sample was measured in duplicate.

4.3.4. Bacteriocin molecular weight

P. pentosaceus DT016 was grown in MRS broth for 18 h at 37 °C. The bacteriocin was precipitated from the cell-free supernatants (Abrams et al., 2011) and proteins separated by tricine-SDS-PAGE (Schagger and von Jagow, 1987). The gels were fixed and one half stained with Coomassie Brilliant Blue. The position of the active bacteriocin was determined by overlaying the other half of the gel (not stained and pre-washed with sterile distilled water) with cells of *L. monocytogenes* 1336 (10^6 CFU ml^{-1}), embedded in TSBYE agar (0.7% w/v). Incubation was at 37 °C for 24 h.

4.3.5. Bacteriocin stability

Cells from an overnight culture of *P. pentosaceus* DT016 were harvested (8000× g, 10 min, 4 °C). The antibacterial activity of the cell-free supernatant was studied, as described by Abrams et al. (2011), after addition of the enzymes proteinase K, pronase, papain, pepsin and trypsin (Boehringer Mannheim GmbH, Mannheim, Germany), α-amylase and catalase (Sigma–Aldrich) and detergents sodium dodecyl sulphate, Tween 20, Tween 80, Triton X-114, Triton X-100, oxbile, EDTA urea and NaCl. The effect of pH and temperature were also investigated (Abrams et al., 2011). All tests were made in triplicate.

4.3.6. Cell lysis and adsorption studies

Cell lysis and adsorption of bacteriocin to producer cells was studied as described by Abrams et al. (2011). All experiments were made in triplicate and each sample was measured in duplicate.

4.3.7. Genes encoding bacteriocin production

Genes encoding bacteriocin production were identified as described by Albano et al. (2007). *P. acidilactici* HA-6111-2 was the positive control strain (Albano et al., 2007). Amplified DNA was purified using the NZYGelpure kit (NZYTech, Genes and Enzymes), and sequenced by MACROGEN. On-line similarity searches were performed with the BLAST program in GenBank (http://www.ncbi.nlm.nih.gov, accessed in January 2015).

4.3.8. Data analysis

A one-way ANOVA was used to assess the influence of the bacteriocin DT016 addition to the *Listeria* cultures growth. Multiple comparisons on mean values *Listeria* enumerations were evaluated by Tukey's post-hoc test using SPSS statistics 22 (IBM, New York, USA). The level of significance for all tests was 0.05.

4.4. Results and Discussion

4.4.1. Identification of bacteriocin-producing LAB strain and bacteriocin production/activity

Growth of the target strains was inhibited by the cell-free supernatant of isolate DT016 and this was associated with an inhibitory compound of proteinaceous nature. Isolate DT016 was identified as *Pediococcus pentosaceus*.

P. pentosaceus DT016 produces a bacteriocin (bacDT016) during exponential growth (Fig. 1). Low levels of bacDT016 activity against *Listeria* population were recorded from 6 h after inoculation, indicating that the peptides are primary metabolites. The highest level of bacteriocin activity was recorded after 15 h of growth. The activity stabilized for the next 3 h and then decreased over the following 6 h (Fig. 1). This decrease was probably due to the effect of extracellular proteases, adsorption to cell surfaces and to feedback regulation (Anastasiadou et al., 2008).

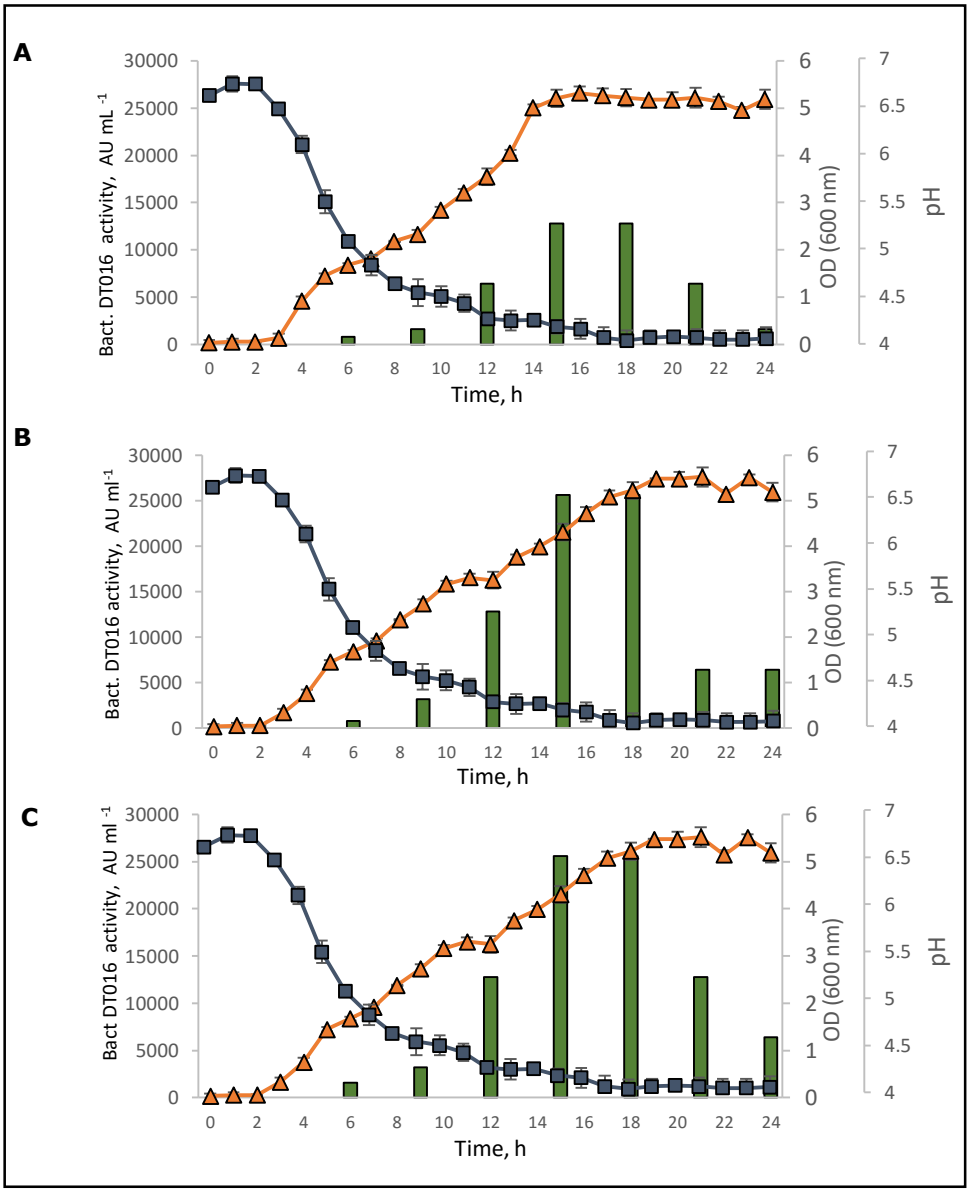

Figure 1. Production of bacteriocin DT016 in MRS broth (pH 6.4) at 37 °C. Antimicrobial activity is presented as AU ml^{-1} (bars). The target strains are for A: *Listeria inoccua* 2030c; B: *L. monocytogenes* 1334; C: *L. monocytogenes* 1336. Changes in optical density (▲) and pH (■) are indicated. Error bars show standard deviation.

4.4.2. Bacteriocin molecular weight

From the association between the zone of growth inhibition and the peptide band position, the active protein was determined (Fig 2). BacDT016 is between 11 and 17 kDa in size; this is higher than most bacteriocins previously described for *Pediococcus* spp.. However, pediocin from *P. acidilactici* PAC1.0, has a molecular weight around 16.5 kDa (Papagianni and Anastasiadou, 2009).

Based on the strong antilisterial activity, it is likely that bacDT016 is a class IIa bacteriocin (Martinez et al., 2013).

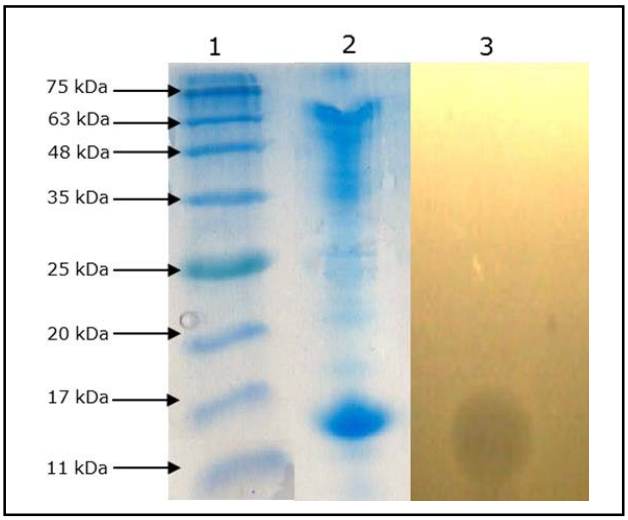

Figure 2. Tricine-SDS-PAGE of bacteriocin DT016. Lane 1: molecular mass marker; lane 2: peptide band stained with Coomassie Blue R250; lane 3: zone of growth inhibition, corresponding to the position of the peptide band in lane 2.

4.4.3. Bacteriocin stability

Treatment of bacDT016 with proteinase K, pronase and trypsin (0.1 and 1.0 mg ml^{-1}) resulted in complete loss of antibacterial activity (Table 1). The addition of papain (0.1 mg ml^{-1} and 1 mg ml^{-1}) and pepsin (1 mg ml^{-1}) reduced the antibacterial activity. Sensitivity was strain dependent.

BacDT016 was resistant to treatment with SDS, Tween 20, Tween 80, oxbile, EDTA, urea and NaCl. However, the bacteriocin showed sensitivity to Triton X-100 and Triton X-114. The bacteriocin remained stable for pH values ranging from 4.0 to 8.0 (Table 1). Antimicrobial activity was strongly reduced at pH values of 2.0 and above 8.0. Similar results have been reported for pediocin AcH/PA-1 (Albano et al., 2007).

Temperatures between 4 and 60 ºC had almost no effect in the bacteriocin activity. Antibacterial activity decreased gradually at temperatures above 60 ºC. Remarkably, antibacterial activity could still be recorded after 15 min at 121 ºC. Similar results have been reported for other bacteriocins produced by *Pediococcus* spp. (Todorov and Dicks, 2009).

Table 1. Effect of treatments on the antibacterial activity of bacteriocin DT016

	Bacteriocin DT016			
		Pathogen		
		L. innocua 2030c	*L. monocytogenes* 1334 (1/2c)	*L. monocytogenes* 1336 (1/2b)
Enzymes	Proteinase K 1.0 and 0.1 mg ml $^{-1}$	100 %	100 %	100 %
	Pronase 1.0 and 0.1 mg ml $^{-1}$	100 %	100 %	100 %
	Papain 0.1 mg ml $^{-1}$	50 %	50 %	50 %
	Papain 1.0 mg ml $^{-1}$	75 %	87.5 %	87.5 %
	Pepsin 0.1 mg ml $^{-1}$	50 %	0 %	0 %
	Pepsin 1.0 mg ml $^{-1}$	75 %	87.5 %	87.5 %
	Trypsin 1.0 and 0.1 mg ml $^{-1}$	100 %	100 %	100 %
	α-amylase 1.0 and 0.1 mg ml $^{-1}$	0 %	0 %	0 %
	Catalase 1.0 and 0.1 mg ml $^{-1}$	0 %	0 %	0 %
Detergents	SDS 0.01 g ml $^{-1}$	0 %	0 %	0 %
	Tween 20 0.01 g ml $^{-1}$	0 %	0 %	0 %
	Tween 80 0.01 g ml $^{-1}$	0 %	0 %	0 %
	Triton X-114 0.01 g ml $^{-1}$	50 %	50 %	50 %
	Triton X-100 0.01 g ml $^{-1}$	50 %	50 %	50 %
	Oxbile 0.01 g ml $^{-1}$	0 %	0 %	0 %
	EDTA 0.1, 2.0 and 5.0 mM	0 %	0 %	0 %
	Urea 0.01 g ml $^{-1}$	0 %	0 %	0 %
	NaCl 0.01 g ml $^{-1}$	0 %	0 %	0 %
pH	2.0	50 %	50 %	50 %
	4.0	0 %	0 %	0 %
	6.0	0 %	0 %	0 %
	8.0	0 %	0 %	0 %
	10.0	75 %	50 %	75 %
	12.0	75 %	75 %	75 %
Temperature	4 ºC	0 %	0 %	0 %
	25 ºC	0 %	0 %	0 %
	30 ºC	0 %	0 %	0 %
	37 ºC	0 %	0 %	0 %
	60 ºC	0 %	0 %	0 %
	80 ºC	25 %	25 %	25 %
	100 ºC	50 %	50 %	50 %
	121 ºC	87.5 %	75 %	75 %

4.4.4. Cell lysis and adsorption studies

Addition of bacDT016 to early-log cultures of the target strains (OD600 nm≈0.1) decreased the growth for 10 h ($p>0.05$) (Figure 3).

The bacteriocin demonstrated bactericidal activity against *Listeria* cells. In the untreated samples (control) *Listeria* cells increased along the 13 h of the study, reaching the stationary phase. At the 12h the maximum load of *L. innocua* 2030c, *L. monocytogenes* strains 1334 and 1336 was 10.4, 10.2 and 10.2 log CFU ml^{-1}, respectively. After 3 h of the bacteriocin application, there was a decrease in *Listeria* cells of about 1.5, 2.2 and 2.2 to *L. innocua* 2030c, *L. monocytogenes* 1334 and *L. monocytogenes* 1336, respectively (Table 2). At the end of the experiment, it was observed a difference on *Listeria* cells growth of ≈5 log CFU ml^{-1} ($p<0.05$) between the samples with no bacteriocin and with bacteriocin added (Table 2).

No bacteriocin activity was detected after treatment of *P. pentosaceus* DT016 with 100 mM NaCl pH 2.0 (data not shown), suggesting that the bacteriocins did not adhere to the surface of the producer cells. Similar results were reported for other bacteriocins (Albano et al., 2007, Ivanova et al., 2000, Todorov and Dicks, 2005).

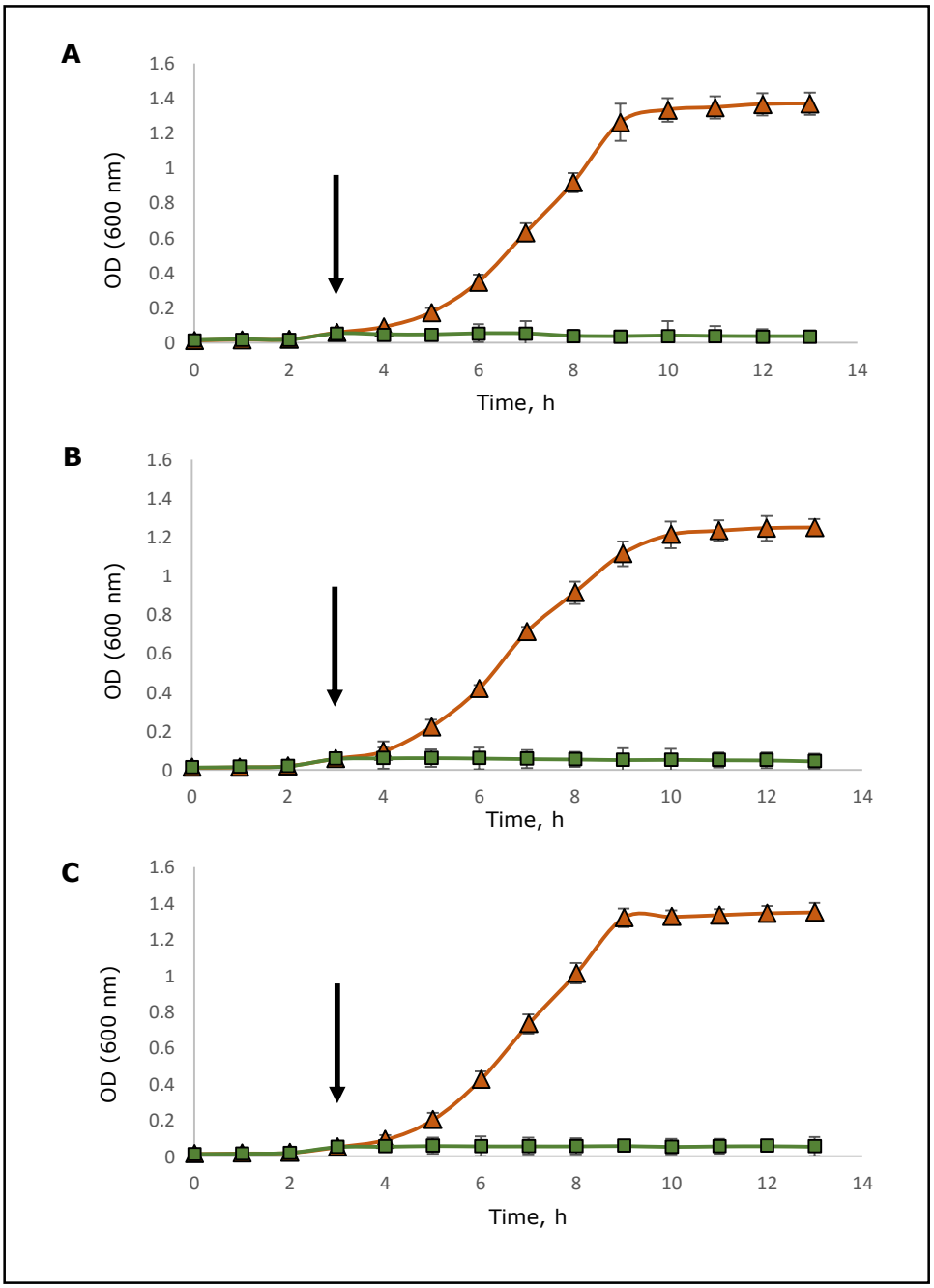

Figure 3. Effect of bacteriocin DT016 on the growth (■) of A: *L. innocua* 2030c; B: *L. monocytogenes* 1334, C: *L. monocytogenes* 1336. The symbol (▲) represents the growth without added bacteriocin (controls). Arrows indicate the point at which the bacteriocin was added (3 h). Error bars show standard deviation.

Table 2. Effect of bacteriocin DT016 on *Listerial* cell growth. SD-Standard deviation

Time (h)	*L. innocua* 2030c (log CFU mL $^{-1}$)				*L. monocytogenes 1*334 serotype 1/2c (log CFU mL $^{-1}$)				*L. monocytogenes* 1336 serotype 1/2b (log CFU mL $^{-1}$)			
	No Treatment	SD	bacteriocin DT016	SD	No Treatment	SD	bacteriocin DT016	SD	No Treatment	SD	bacteriocin DT016	SD
0	7.2	0.08	7.3	0.09	6.9	0.20	7.3	0.22	7.6	0.03	7.8	0.18
3	8.3	0.08	8.3	0.13	8.3	0.08	8.2	0.11	8.2	0.10	8.2	0.11
6	8.6	0.06	6.8	0.14	8.9	0.09	6.0	0.17	8.8	0.09	6.0	0.11
9	9.8	0.14	5.3	0.12	9.7	0.08	5.2	0.10	10.0	0.04	5.6	0.10
12	10.4	0.11	5.2	0.09	10.5	0.15	5.2	0.16	10.2	0.12	5.0	0.16

4.4.5. Genes encoding bacteriocin production

P. pentosaceus DT016 has a 1110 bp DNA fragment with homology to pediocin AcH/PA-1 (Genbank accession number NG_035882.1, (Miller et al., 2005)). Bacteriocin DT016 is thus considered similar to pediocin AcH.

4.4.6.Biopreservation potential

The LAB culture, *P. pentosaceus* DT016, described in this study shows promising potential as a protective culture to inhibit and reduce *L. monocytogenes* proliferation in vegetables. In fact, the effective inhibition of *L. monocytogenes* by the application of *P. pentosaceus* strains as starter culture to various foods has been demonstrated (Huang et al., 2009, Jang et al., 2015, Kingcha et al., 2012). On other hand, the bacteriocin produced is homologous to pediocin AcH, which have been extensively studied and is considered a good biopreservative agent (Miller et al., 2005, Nieto-Lozano et al., 2010). Therefore, *P. pentosaceus* DT016 and pediocin DT016 have the potential to be used in effective biocontrol approaches towards *L. monocytogenes*.

4.5. Conclusions

Bacteriocin bacDT016 produced by a strain of *P. pentosaceus* originally isolated from Iceberg lettuce exhibits activity against *L. monocytogenes* and *L. innocua*. Antilisterial activity was traced to a heat-resistant 11 to 17 kDa protein similar to pediocin AcH. In addition, bactDT016 is stable in a wide range of pH and maintains the antilisterial activity at refrigeration temperature (4 ºC).

In conclusion, *P. pentosaceus* DT016 has the potential to be used as a bioprotective culture in minimally processed vegetables and fruits and the antibacterial compound

produced may improve the safety and shelf-life of these products, while answering the need for effective biopreservation techniques.

4.6. Acknowledgements

This work was supported by National Funds from FCT – Fundação para a Ciência e a Tecnologia through project PEst-OE/EQB/LA0016/2013.

Financial support for Ramos B., Ferreira, V. and Brandão T.R.S. was provided by FCT and Fundo Social Europeu (FSE) through fellowships SFRH/BD/42169/2007, SFRH/BPD/72617/2010 and SFRH/BPD/41419/2007, respectively.

Collaboration of Diana Silva and Tatiana Esteves in the experimental procedures is gratefully acknowledged.

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Chapter 5

Biopreservation approaches to reduce Listeria monocytogenes in fresh vegetables

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Submitted to *Food Microbiology (October, 2015)*.

5.1. Abstract

The survival of *Listeria monocytogenes* was evaluated along time in fresh lettuce, rocket salad, parsley and spinach and in these vegetables after the addition of *Pediococcus pentosaceus* DT016 as a protective culture. The green vegetables were inoculated with a *L. monocytogenes* cocktail at levels of about 6 and 4 log CFU/g and stored at 4 ºC. The pathogen load in the presence of the protective culture decrease along storage. Contrary to that, in the fresh vegetables *L. monocytogenes* was able to grow and an increasing load was observed during refrigerated storage. At the last day of storage, the presence of the protective culture resulted in a minimum pathogen reduction of 1.4 log CFU/g when compared with the pathogen load in fresh vegetables. *Pediococcus pentosaceus* DT016 showed to be a promising alternative to maintain the safety of fresh vegetables kept under cold storage.

In other experiment, the potential use of pediocin DT016, the bacteriocin produced by *P. pentosaceus* DT016, was studied in a washing step for fresh vegetables. Fresh lettuce, rocket salad, parsley and spinach were contaminated with *L. monocytogenes* and the pathogen load was studied, after washing with: water, a commercial sodium hypochlorite solution (AMUKINA) and the pediocin solution, along storage at 4 ºC. It was observed that the pediocin solution immediately after washing significantly reduced the initial *L. monocytogenes* load and along storage inhibited the pathogen proliferation. In the vegetables washed with water and AMUKINA the pathogen was able growth along storage. At the end of storage, the pathogen load in the vegetables washed with the pediocin was lower than in the vegetables washed with water and AMUKINA, by a minimum of 3.2 and 2.7 log CFU/g, respectively. Therefore, the application of washing solutions containing pediocin DT016 is a very promising method to reduce and inhibit *L. monocytogenes* proliferation in fresh vegetables at refrigerated storage.

Keywords: *Listeria monocytogenes, Pediococcus pentosaceus* DT016, fresh vegetables, protective culture, pediocin DT016, washing step.

5.2. Introduction

Listeria monocytogenes is considered ubiquitous in the agricultural environment and its occurrence in vegetables may be as high as 25% (Cordano and Jacquet, 2009, Crepet et al., 2007, Samara and Koutsoumanis, 2009, Sant'Ana et al., 2012). In fact, raw vegetables have been identified as a vehicle of transmission of foodborne outbreaks and play an important role in listeriosis epidemiology (Allende et al., 2007, Beuchat, 2002, Luber et al., 2011, Ramos et al., 2014). This is of special concern because this kind of foods are eaten raw and rely only on cold storage to maintain their safety, however *Listeria* has the ability to survive and multiply at refrigeration temperatures (Ramos et al., 2014).

Currently, commercial operations use wash treatments with antimicrobials as the only step to reduce microbial populations on fresh produce, where chlorine is the most commonly used sanitizer. However, numerous reports indicate that chlorine has limited antimicrobial efficacy at the permitted levels and has been associated with the production of potentially toxic substances (São José et al., 2014, Siroli et al., 2015b).

Consumers' concern about chemical residues in food products has led the food industry to seek novel and alternative technologies to improve food quality and safety with the minimal use of chemicals (Alegre et al., 2013). The use of generally recognized as safe (GRAS) microorganisms such as lactic acid bacteria (LAB) and/or their natural metabolites shows to be a promising alternative to maintain the food safety and it is also perceived by the consumer as a natural food preservation method (Engelhardt et al., 2015, Ramos et al., 2013).

Protective cultures of LAB have been developed over the last few decades to increase the safety and shelf-life of fresh and minimally processed vegetables (Siroli et al., 2015a, Siroli et al., 2015b, Trias et al., 2008a, Vescovo et al., 1996). The preservation abilities of LAB are a result of several mechanisms of action and are mainly related to the production of antimicrobial compounds, organic acids, hydrogen peroxide, bacteriocins and diacetyl (Allende et al., 2007). Moreover, they compete with pathogens and spoilage microorganisms for nutrients (vitamins, minerals, trace elements and peptides).

Bacteriocins, ribosomally synthesized antimicrobial peptides, are able to kill or inhibit the growth of other bacteria, and are considered to be safe natural biopreservatives.
The direct application of bacteriocins in fresh cut products has been tested in recent years. Several authors showed a reduction in *L. monocytogenes* loads of 1.2 to 2.7 log units in vegetables when washed with bacteriocin solutions (Allende et al., 2007, Molinos et al., 2005, Randazzo et al., 2009, Siroli et al., 2015a).
In this study, a bacteriocinogenic LAB previously isolated from lettuce, with antibacterial activity towards *L. monocytogenes* and *L. innocua,* was used. The strain, identified as *Pediococcus pentosaceus* DT016, produces a bacteriocin that is identical to pediocin PA-1/AcH (Miller et al., 2005). The aim was to investigate the potential use of *P. pentosaceus* DT016 and its bacteriocin as biopreservation agents for green vegetables. The ability of *P. pentosaceus* to supress *L. monocytogenes* under refrigerated storage was assessed in fresh vegetables. Additionally, it was determined whether it would be possible to use pediocin DT016, the bacteriocin produced by *P. pentosaceus*, as a biopreservation factor in a washing step for fresh or minimally processed vegetables.

5.3. Material and Methods

5.3.1. Vegetables

Iceberg lettuce (*Lactuca sativa capitata*), rocket salad (*Eruca sativa* Mill.), spinach (*Spinacia oleracea* L.) and parsley (*Petroselinum crispum* Mill.) were purchased in a local supermarket the day before the experiment. The vegetables were washed in running tap water for 2 min to remove soil particles, let too dry at room temperature and kept at 4 ºC until initiation of experiments. When applicable, prior to the washing step, the vegetables outer layers were removed and the leaves were portioned by hand.

5.3.2. Bacterial strains, media and culture conditions

The bacterial strains used in this work were: *Pediococcus pentosaceus* DT016 and three-strain composite of *Listeria monocytogenes.*

Pediococcus pentosaceus DT016 was previously isolated from lettuce (data not shown) and was used as a protective culture or as pediocin DT016 producer.

The LAB strain was grown for 24 h at 37 ºC in de Man, Rogosa Sharpe broth (MRS, Lab M, Lancashire, UK). The *Listeria* strains: 1334 serotype 1/2c, 1336 serotype 1/2b, and 1092 serotype 4b (Escola Superior de Biotecnologia, UCP) were grown independently for 24 h at 37 ºC in Tryptic Soy Broth (TSB, Lab M) with 0.6% yeast extract -TSBYE (Lab M).

P. pentosaceus DT016 second subculture was incubated in MRS broth (Lab M) at 37 ºC for 24 h and washed twice by centrifugation (5000 rpm, 15 min, 4 ºC) with sterile distilled water. The final cell pellets were resuspended in distilled water so that the final cell numbers in the suspension were approximately 8 log CFU/mL.

Two consecutive subcultures of *L. monocytogenes* strains were prepared in 5 ml TSBYE (Lab M) with a final transfer of 1 ml to 100 ml of the same media. Cultures were incubated at 37 °C for 24 h and then washed, as mentioned before, with sterile distilled water. The three pathogen cultures were mixed together in the same proportion (1:1:1), and the cell pellets were resuspended in distilled water to obtain final bacterial concentrations of about 5 and 7 ranging log CFU/mL.

5.3.3. Preparation of the washing solutions

A sodium hypochlorite solution (NaOCl, 200 μg/mL; recommended concentration for produce washing) was prepared from a commercial bleach disinfectant available for fresh fruits and vegetables (1.15 mg/mL – AMUKINA, Farma-Lepori), and according to manufacturer's instructions.

Pediocin DT016 was obtained by cultivation of the producer strain, *P. pentosaceus* DT016, in MRS broth (Lab M) overnight at 37 ºC. Then the culture was centrifuged (5000

rpm, 15 min, 4 ºC), the cell-free supernatant was filtered sterilized (0.22 µm, Corning Incorporated, Germany) and the pH adjusted to 6.5 with NaOH (1 N). The pediocin DT016 washing solution was prepared by diluting 200 mL of the bacteriocinogenic supernatant in 1800 mL of sterile distilled water.

Additional experiments using water washings (sterile distilled water) were performed as control.

All solutions were prepared immediately before use.

5.3.4. Effect of Pediococcus pentosaceus DT016 against Listeria monocytogenes populations inoculated in fresh vegetables

Pathogen concentrated suspensions were obtained as described previously. Antagonist, *P. pentosaceus* DT016, was tested at 7 log CFU/mL and *L. monocytogenes* at 6-7 (L_1) and 4 (L_2) log CFU/mL.

The vegetables were immersed in suspensions containing the pathogen alone (L_1 and L_2), or the antagonist and the pathogen ($P+L_1$, $P+L_2$), for 15 min and placed on sterile paper for removing excess liquid at room temperature (20 ºC). Vegetables exclusively inoculated with *P. pentosaceus* DT016 were used to analyse the antagonist fate in the vegetables. To facilitate the attachment of bacteria, samples were stored for 1h at 4 ºC and then aseptically divided into 50 g portions and placed in sterile bags for storage over a 15-day period at 4 °C.

Pathogen, LAB and mesophilic bacteria levels were determined immediately after inoculation and along storage at 4 °C.

All experiments were made in triplicate.

5.3.5. Effect of washing solutions on Listeria monocytogenes populations inoculated in fresh vegetables

The vegetables were contaminated with the *L. monocytogenes* cocktail suspension as described by Ramos et al. (2014) .

Approximately 500 g of each inoculated vegetable (lettuce, rocket salad, spinach and parsley) were washed for 5 min, at room temperature, in 2 L of the following solutions: (1) sterile distilled water (H_2O), (2) sodium hypochlorite 200 µg/mL solution (AMUKINA), (3) pediocin DT016 solution (Pediocin).

After washing, the vegetables were placed on sterile absorbent paper to allow removal of the excess liquid at room temperature (20 ºC). The samples were aseptically divided into 50 g portions and placed in sterile bags for storage over a 15-day period at 4 °C.

Listeria, Lactic acid bacteria and mesophilic bacteria enumeration was done before and immediately after the washing procedures and along the refrigerated storage.

All experiments were made in triplicate.

5.3.6. Microbiological analyses

The vegetable samples with the pathogen and those supplemented with LAB were analysed immediately after the treatment and along the produce shelf-life. For lettuce samples the analysis were performed along 7 days of storage (0, 3, 5 and 7 days); for rocket salad the analysis were performed 9 days of storage (0, 3, 5, 7 and 9 days), whereas for spinach and parsley the samples were evaluated along 15 days of storage (0, 3, 5, 7, 9, 12 and 15 days). In the trials involving the vegetables washings, the analyses were performed before and immediately after treatment and along storage. The sampling period for each vegetable was the same as for the experiment mentioned above. The control samples not inoculated with the target pathogenic species were checked for the presence of naturally occurring *L. monocytogenes*.

Before and after treatments and during storage, 25 g samples of each vegetable were aseptically transferred to 225 mL of Buffered Peptone Water - BPW (Lab M) and homogenized in a Stomacher (Lab-Blender 400, Seward Medical, London, UK) for 90s. Each sample was serially diluted and plated in duplicate onto Tryptic Soy Agar-TSA (Lab M) to enumerate the mesophilic bacteria (30 ºC, 48 h), onto MRS agar (Lab M) to enumerate the LAB population (37 ºC, 48 h) and Palcam agar (Merck) plus selective supplement (Merck) to estimate *L. monocytogenes* population size (37 ºC, 48 h). The non-

Listeria populations were calculated as the difference between TSA counts and viable counts of *L. monocytogenes* on Palcam agar.

Mean values of bacterial counts (CFU/g), from duplicate plate samples, were converted to log numbers for each combination.

The bacteriocinogenic activity in the vegetable samples was also analysed. Samples (10 g each) of fresh vegetables were homogenized in sterile distilled water and when required the pH was adjusted to 6 with 0.02 N HCl, and centrifuged at 5000 rpm for 15 min. The supernatants were filtered sterilized (0.22 μm, Corning Incorporated, Germany) and the inhibitory activity was assayed by the agar-spot test. Briefly, the *L.* monocytogenes strains (mentioned in section 2.2.) were grown overnight at 37 ºC in TSBYE (Lab M) and evenly spread with a sterile cotton swab in TSAYE (Lab M). Plates were allowed to set and 20 μL drops of vegetables samples were spotted on the lawns of pathogens. Plates were incubated at 30 ºC until confluent growth was observed and then examined for zones of inhibition around the inoculation spots.

5.3.7. Colour properties

The colour values (*L**, *a** and *b**) of fresh and minimally processed vegetables were assessed using a Minolta CR-400 colorimeter (Konica-Minolta, Osaka, Japan), calibrated with a white standard tile. Lightness value *L** indicates how dark/light the sample is (ranges from black at 0 to white at 100). The chromaticity coordinate *a** measures red when positive and green when negative, and the chromaticity coordinate *b** measures yellow when positive and blue when negative.

Analysis was carried out on 10 pieces of each vegetable randomly chosen from each bag at 3, 5, 7, 9, 12 and 15 days of storage at 4 ºC. As mentioned before, the measures limit for lettuce and rocket salad was at the 7^{th} and 9^{th} day of storage, respectively.

5.3.8. pH measurement

Five-gram sample of vegetable tissue was blended with an ultra-turrax homogenizer (Ika digital T25, IKA®-Werke GmbH & Co. KG, Staufen, Germany) for 5 min in 20 mL of ultra-pure water. The pH of the slurry was measured at room temperature using a research pH-meter (GLP 22, Crison Instruments, Barcelona, Spain).

5.3.9. Data analysis

A one-way ANOVA was used to assess the influence of the protective culture on the *Listeria monocytogenes*, mesophilic and LAB load and colour properties along storage. The influence of the washing solutions on *Listeria* growth, mesophilic and LAB load and on colour coordinates were also evaluated. Multiple comparisons on mean values of bacteria enumerations and colour coordinates were evaluated by Tukey's post-hoc test using SPSS statistics 22 (IBM, New York, USA). The level of significance for all tests was 0.01.

5.4. Results and Discussion

5.4.1. Effect of Pediococcus pentosaceus DT016 against Listeria monocytogenes populations inoculated in fresh vegetables

The effect of *in situ P. pentosaceus* DT016 on the behaviour of *L. monocytogenes* in fresh vegetables was determined during the storage period at 4 °C (Figure 1).

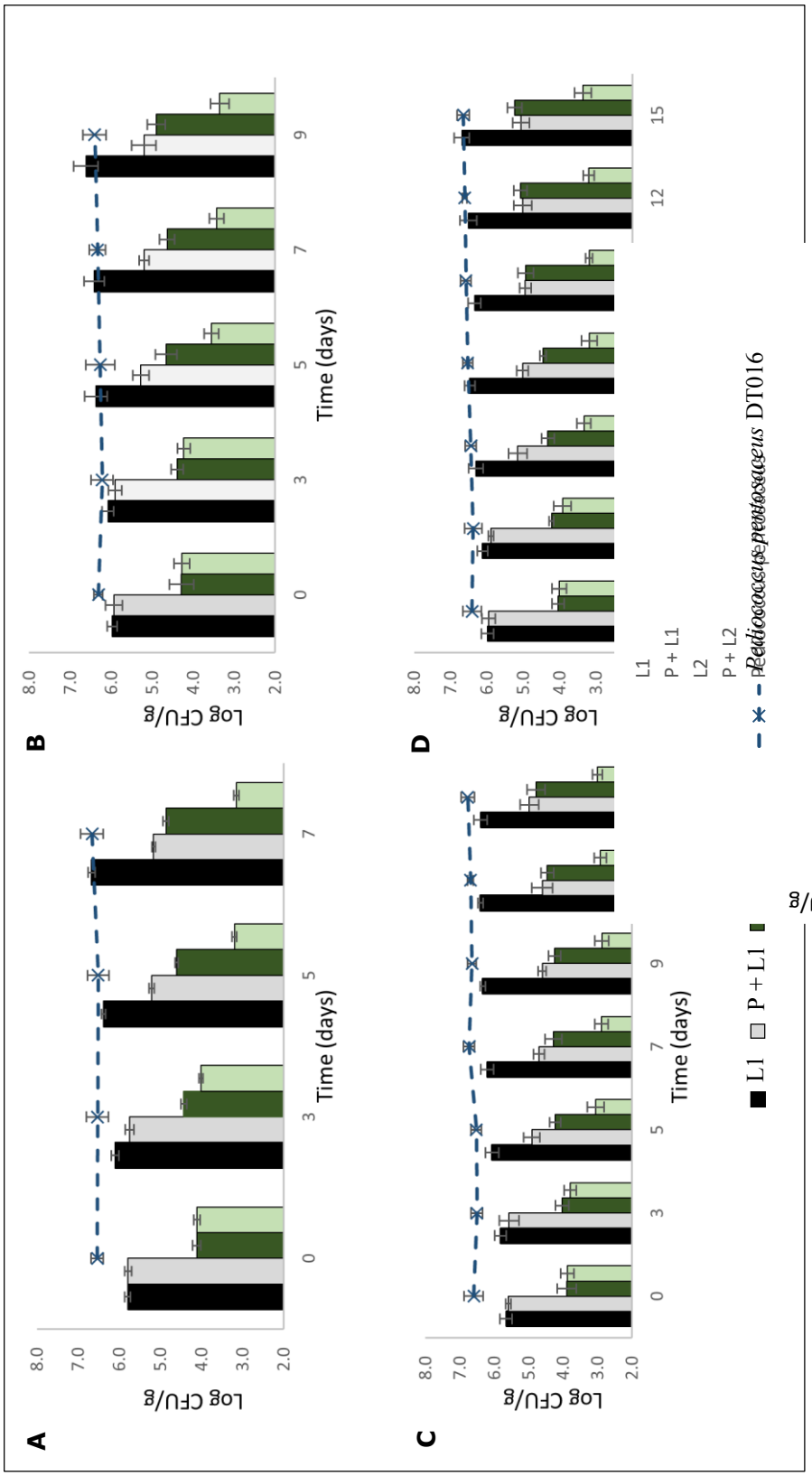

Figure 1. Survival of *Listeria monocytogenes* i
as a protective culture (P+L_1 and P+L_2), duri
represented by the line – ✱ – The vegetables
deviation (SD).

occus pentosaceus DT016
getables, along storage, is
Error bars show standard

Pediococcus pentosaceus DT016 was able to survive and grow in all the vegetables at the selected temperature. When inoculated alone the LAB counts were above 7 log CFU/g in all the vegetables during the storage period. In the presence of the pathogen, *P. pentosaceus* maintains levels above 6.5 log CFU/g along the storage period in all the vegetables studied (Figure 1).

During storage and for experiments L_1 (initial load of 6-7 log CFU/mL), in the absence of the protective culture, *L. monocytogenes* grew throughout the shelf life period of all the vegetables, reaching large numbers of 6.7, 6.6, 6.4 and 6.7 log CFU/g in lettuce, rocket salad, parsley and spinach, respectively. *Listeria monocytogenes* counts were reduced in all the vegetables inoculated with the protective culture. At the end of storage, a difference in *L. monocytogenes* population by 1.5, 1.4, 1.4 and 1.6 log units was respectively observed in lettuce, rocket salad, parsley and spinach with *P. pentosaceus* and in the fresh vegetables without protective culture.

In a separate experiment, a lower inoculum of *L. monocytogenes* (L_2) was tested with similar outcome, although the *Listeria* reduction achieved being superior ($p<0.01$) (Figure 1).

In the present study, the presence of antibacterial activity in all the vegetables was detected at the 3th day of storage, by the direct application of filtered vegetables samples to *L. monocytogenes* (data not shown). The high antimicrobial activity in vegetables detected at 3th day is probably mainly due to bacteriocin production during storage and cannot be attributed to a reduction in pH or production of lactic acid as the pH values (data not shown) remain identical to the vegetables in the absence of the protective culture ($p>0.01$).

Our results are in agreement with other studies. Cai et al. (1997) found that the bacteriocin producing *Lactococcus lactis* subsp. *lactis* isolated from bean sprouts was able to reduce *L. monocytogenes* counts in ready to eat Caesar salad by 1 to 1.4 logs, after storage for 10 days at 7 and 10 ºC. In alfalfa sprouts the addition of *Lactococcus lactis* as a protective culture resulted in *L. monocytogenes* inhibition of 1 log unit (Palmai and Buchanan, 2002). Other studies proved the efficacy of LAB cultures towards *L. monocytogenes* growth in scarola leaves (Scolari and Vescovo, 2004), in samples of fresh fruit and vegetables (Trias et al., 2008a, Trias et al., 2008b), in minimally processed kale (Costa et

al., 2009) and in ready to eat artichoke products (Valerio et al., 2013). Recently, Siroli et al. (2014) revealed the effectiveness of a nisin producing strain *L. lactis* CBM21, inoculated in the washing solution of minimally processed lamb's lettuce, to inhibit *L. monocytogenes*. Moreover, Siroli et al. (2015b) showed that applying *Lactobacillus plantarum* V7B3 to lettuce during the washing phase can increase its shelf-life and reduce viability of *L. monocytogenes.*

5.4.2. Effect of washing solutions on Listeria monocytogenes populations inoculated in fresh vegetables

Lettuce samples artificially contaminated with *L. monocytogenes* still retained approximately 6.0 and 5.4 log CFU of viable *Listeria*/g, respectively after washing with water and the AMUKINA solution (sodium hypochlorite 200 µg/mL) (Figure 2 A). In samples treated with pediocin DT016 solution the level of viable *Listeria* after washing was 2.1 and 1.6 log CFU/g lower than the vegetables treated with water and AMUKINA, respectively ($p<0.01$).

Listeria counts increased throughout storage in the lettuce samples treated with water and AMUKINA, reaching large numbers of 7.0 and 6.3 log CFU/g, respectively. In the lettuce samples treated with the pediocin, the pathogen counts decreased along storage until the day 7, when it was observed a slight increase (Figure 2 A). At the end of storage, the washing with the pediocin resulted in a pathogen load significantly inferior than the other washings ($p<0.01$). The reductions achieved were 3.2 and 2.7 log CFU/g higher than the water and AMUKINA washings, respectively.

The rocket salad washing procedure was able to remove in some degree the pathogen from the vegetables. Immediately after washing, it was observed a reduction in *Listeria* counts of 0.2, 0.7 and 2.0 log CFU/g for water, AMUKINA and pediocin ($p<0.01$), respectively. *Listeria monocytogenes* increased along storage in rocket salad washed with water and AMUKINA, reaching large numbers of 7.5 and 6.9 log CFU/g, respectively (Figure 2 B). The pediocin washing inhibited the pathogen proliferation along storage

until the day 7 (Figure 2 B). For all the washings, the maximum pathogen load was achieved at the last day of refrigerated storage. *Listeria* counts were lower in the samples treated with pediocin by 3.0 and 2.1 log CFU/g than the ones treated with water and AMUKINA (p<0.01).

Listeria monocytogenes was reduced in parsley by the washing step with water, AMUKINA and pediocin by 0.6, 1.4 log and 2.9 log CFU/g, respectively (p<0.01). As shown in Figure 2 C, the pathogen was able to survive and grow during storage in parsley washed with water and AMUKINA, reaching levels of 6.0 and 5.2 log CFU/g, respectively. For parsley treated with the pediocin, viable *Listeria* were always below detection limits (2 log CFU/g) from the washing until the 9th day of storage (Figure 2 C). From the 9th day to the end of storage, low levels of the pathogen were detected with a maximum load of 3.1 log CFU/g achieved at the end of storage. *Listeria* counts of pediocin treated samples were significantly lower along the storage period than the parsley washed with the other solutions (p <0.01).

The washing of contaminated spinach led to different *L. monocytogenes* reductions (p<0.01), in the order of 0.2, 0.8 and 2.2 log CFU/g for water, AMUKINA and pediocin solutions, respectively. During the refrigerated storage the pathogen counts increased in the spinach washed with water and AMUKINA (Figure 2 D). For the spinach washed with pediocin, the pathogen load continuous to decrease along storage until the day 9. *Listeria* counts were always lower in the spinach treated with pediocin (p<0.01) and at the end of storage there was a difference in the pathogen population of about 2.5 and 1.9 log CFU/g between the samples washed with the pediocin and samples treated with water and AMUKINA, respectively.

L. monocytogenes counts reduced in all the vegetables treated with pediocin DT016 immediately after washing, and continuously decreased during storage until the 7th day for lettuce and rocket salad and until the 9th day for parsley and spinach.

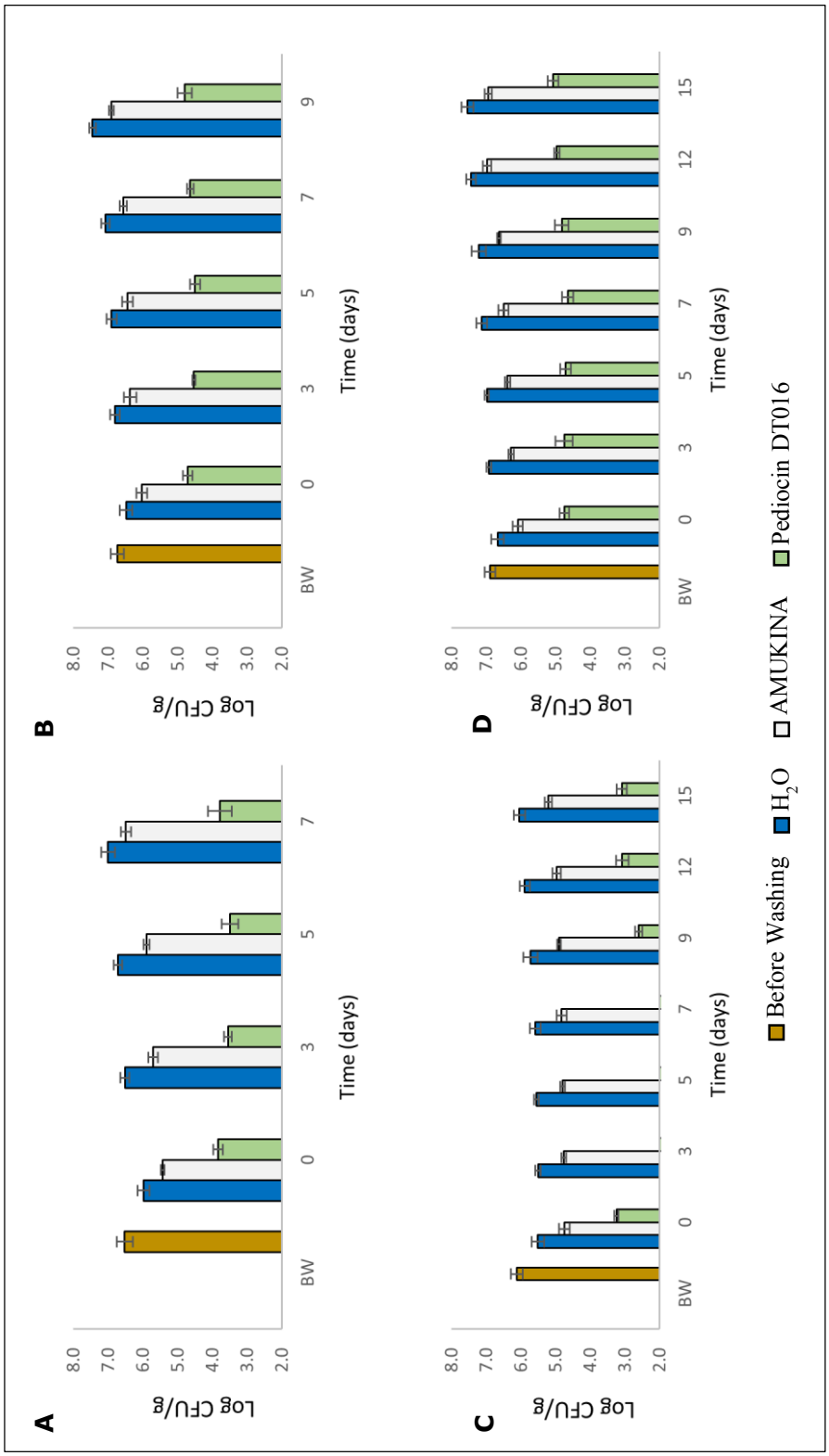

Figure 2. *Listeria monocytogenes* population present in fresh vegetables before washing (BW) and after washing with water, AMUKINA and pediocin DT016 solution, during storage at 4 ºC. The vegetables are A: Lettuce; B: Rocket salad; C: Parsley; D: Spinach. Error bars show standard deviation (SD).

The continuous decrease in the pathogen load observed during storage of the vegetables washed with the pediocin solution, suggests that the adsorbed bacteriocin was still able to inhibit *Listeria* during storage. Actually, it was found that the pediocin washing solution showed residual activity (200 AU/mL) until the 7th day of storage.
The pediocin was unable to completely eliminate *L. monocytogenes* during prolonged storage. This could be due to the interaction with particular food components that may influence the adsorption of the bacteriocin into the vegetables surface and even cause their inactivation. *Listeria* populations were comparatively lower in vegetables after washing with the pediocin solution than in vegetables washed with water or AMUKINA ($p<0.01$). For long storage, the pathogen reductions achieved with the bacteriocin were always higher by a minimum of 2.5 and 1.9 log CFU/g than water and AMUKINA ($p<0.01$), respectively. It should be noted that the pH values of the vegetables treated with the pediocin was similar to the vegetables washed with water ($p>0.01$) (data not shown).

Other studies with bacteriocin solutions reported similar *Listeria* reductions to the results obtained in this study. Bari et al. (2005) found that nisin and pediocin, individually or in combination, resulted in a reduction between 2-4 log units of *L. monocytogenes* in fresh cut cabbage, broccoli and mung bean sprouts. Also, immersion solutions containing enterocin AS-48 reduced *L. monocytogenes* loads below detection limits in sprouts at days 1 to 7 at 6 ºC and 15 °C, and in green asparagus at 15 °C (Molinos et al., 2005). In addition, similar outcomes were obtained by testing the effect of washing with bacteriocin solutions: nisin+, coagulin+ and a nisin–, coagulin+ cocktail (Allende et al., 2007), with bacteriocin RUC9 (Randazzo et al., 2009) and with Enterocin 416K1 (Anacarso et al., 2011) on the survival and proliferation of *L. monocytogenes* in fresh vegetables stored at 4 °C. In summary, the washing step immediately decreased the viability of *L. monocytogenes*, however it was not effective in removing completely the pathogen from the produce.

A promising observation is that washing with the pediocin solution did not significantly affect survival and proliferation of the non-*Listeria* populations present in all the vegetables. In fact, their load is comparable with the water washing ($p>0.01$) (see Figure

3). The AMUKINA solution resulted in lower mesophilic and LAB loads than the other solutions ($p<0.01$). Natural microbiota present in fresh vegetables can play an important role in maintaining their quality and safety. Some authors suggested that the success of sanitation procedures used to eliminate pathogenic bacteria from foods may encourage the emergence of *L. monocytogenes* and other foodborne pathogen organisms by reducing the competitive microorganism populations. In this respect, it should be desirable to sanitize with solutions that preserve these microorganisms (Allende et al., 2007, Ramos et al., 2013).

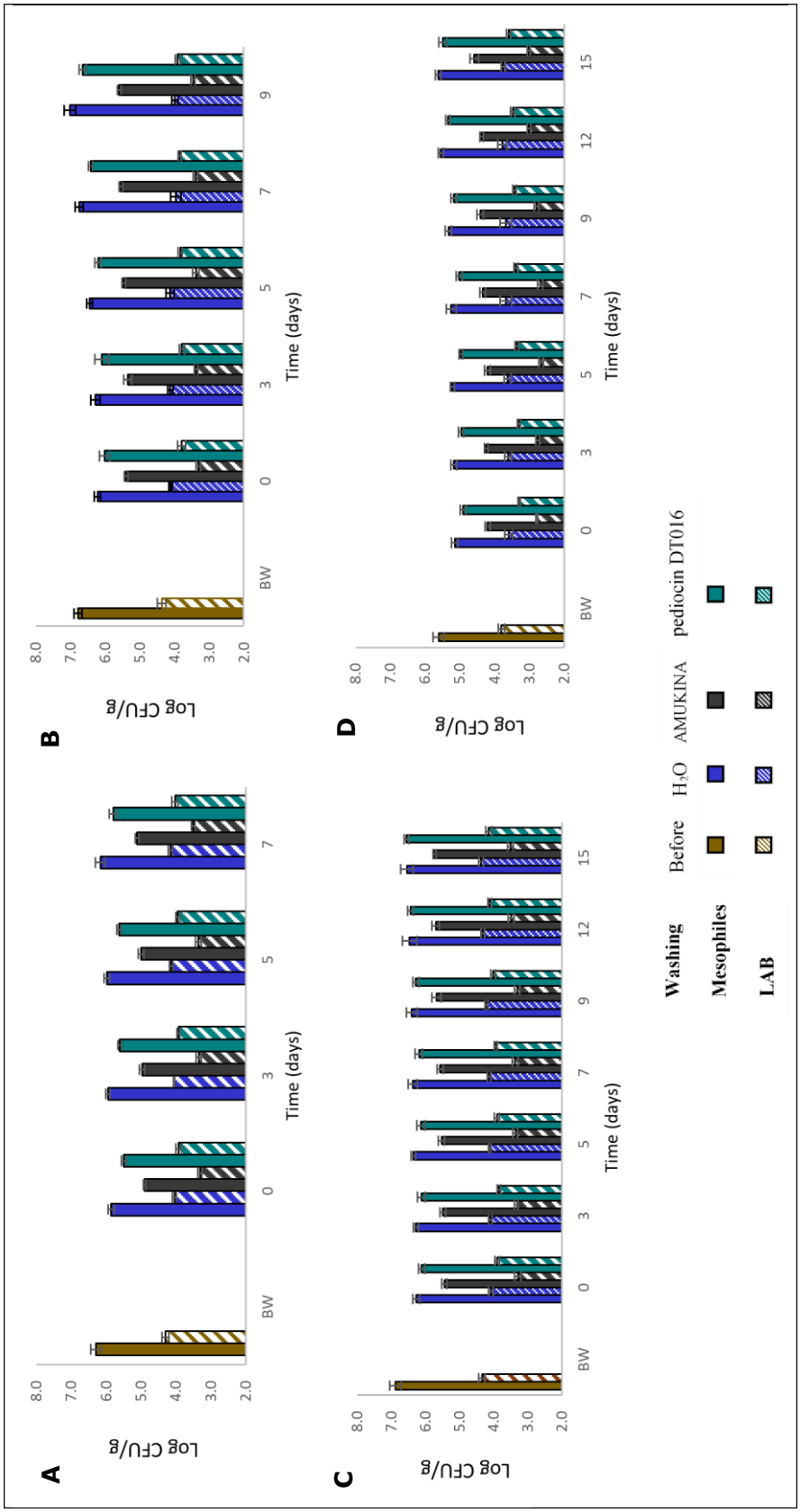

Figure 3 Mesophilic and lactic acid bacteria (LAB) populations present in fresh vegetables before washing (BW) and after washing with water, AMUKINA and pediocin DT016 solution, during storage at 4 ºC. The vegetables are A: Lettuce; B: Rocket salad; C: Parsley; D: Spinach. Error bars show standard deviation (SD).

5.4.3. Colour

5.4.3.1. Colour properties of fresh vegetables inoculated with *Pediococcus pentosaceus* DT016

Colour is one of the first quality characteristics evaluated by consumers. Changes in colour were quantified in lettuce, rocket salad, parsley and spinach. The colour coordinates of fresh vegetables and vegetables with the protective culture, along refrigerated storage, are shown in Figure 4.

No noticeable changes in the colorimeter analyses were observed between the samples off lettuce, rocket salad and parsley ($p>0.01$). With prolonged storage, the spinach samples with the protective culture presented a lower a^* and a higher b^* than the fresh spinach ($p<0.01$). There was an increase in green colour coupled with an increase in yellow aspect of these samples. This change in colour can be due to the metabolites excreted during LAB growth.

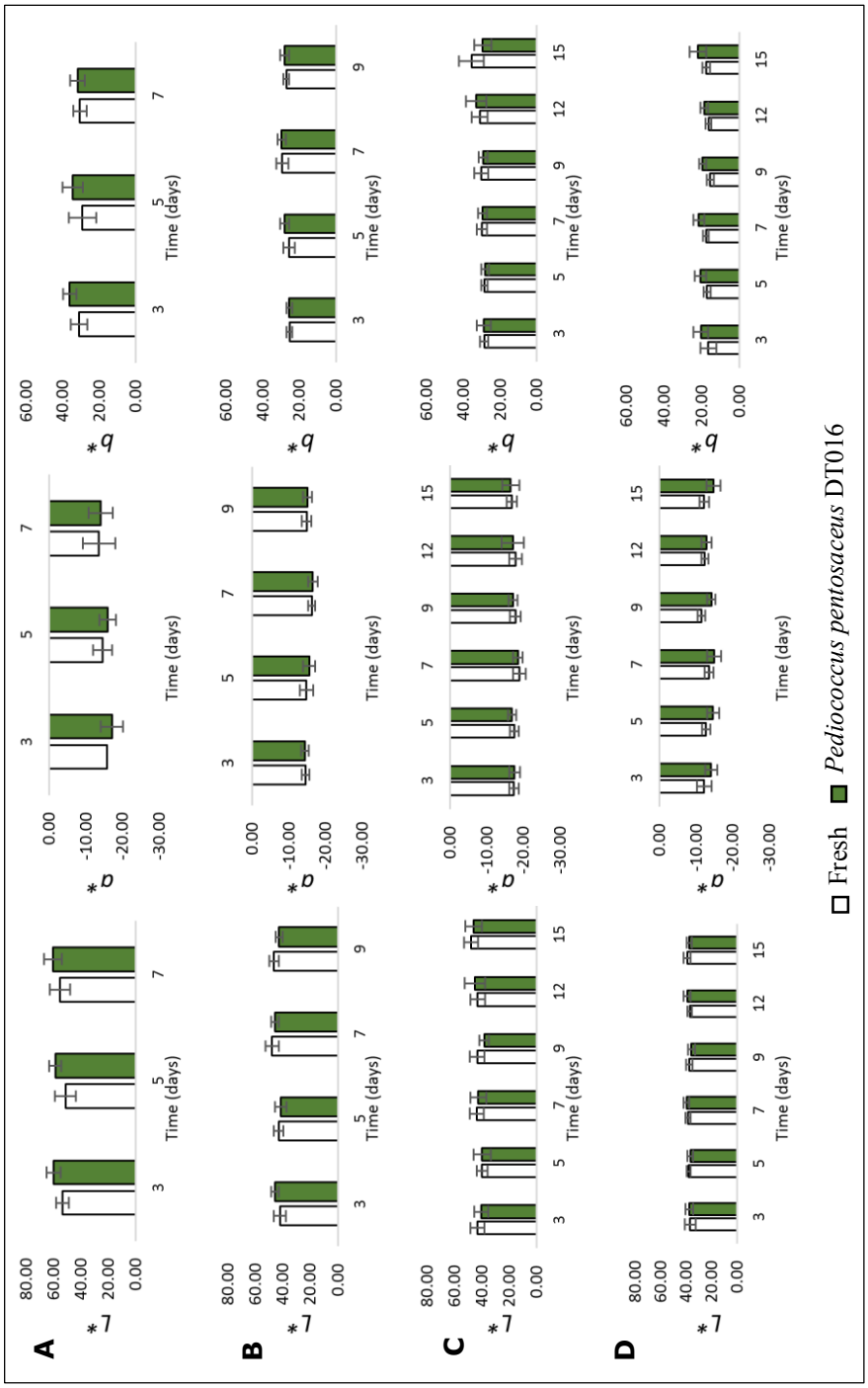

Figure 4. Colour coordinates of fresh vegetables and vegetables with *Pediococcus pentosaceus* DT016, as a protective culture, along storage at 4 ºC. The vegetables are A: Lettuce; B: Rocket salad; C: Parsley; D: Spinach. Error bars show standard deviation (SD).

5.4.3.1. Colour properties of fresh vegetables washed with various solutions

Changes in colour were quantified in lettuce, rocket salad, parsley and spinach after washing with water, AMUKINA and pediocin DT016 along refrigerated storage. The colour coordinates for the treated vegetables along refrigerated storage, are shown in Figure 5.

Lettuce, rocket salad and spinach samples presented similar colour properties after the washing step, independently of the washing solution ($p>0.01$). Along storage the colour coordinates maintain similar values, however, at the last day of storage there were differences between the lettuce samples washed with the pediocin and AMUKINA. AMUKINA washing resulted in a loss of the green colour of the lettuce when compared with pediocin washing ($p<0.01$). This can be due to possible chemical reactions of the vegetable with chlorine sub products.

For parsley samples, washing with AMUKINA and the pediocin resulted in a loss of green colour when compared with the water washing ($p<0.01$). Also, the samples washed with AMUKINA showed an increase in yellow colour when compared with the others ($p<0.01$). Along storage these differences were reduced and at the end of storage, parsley washed with the various solutions presented similar colour properties ($p>0.01$).

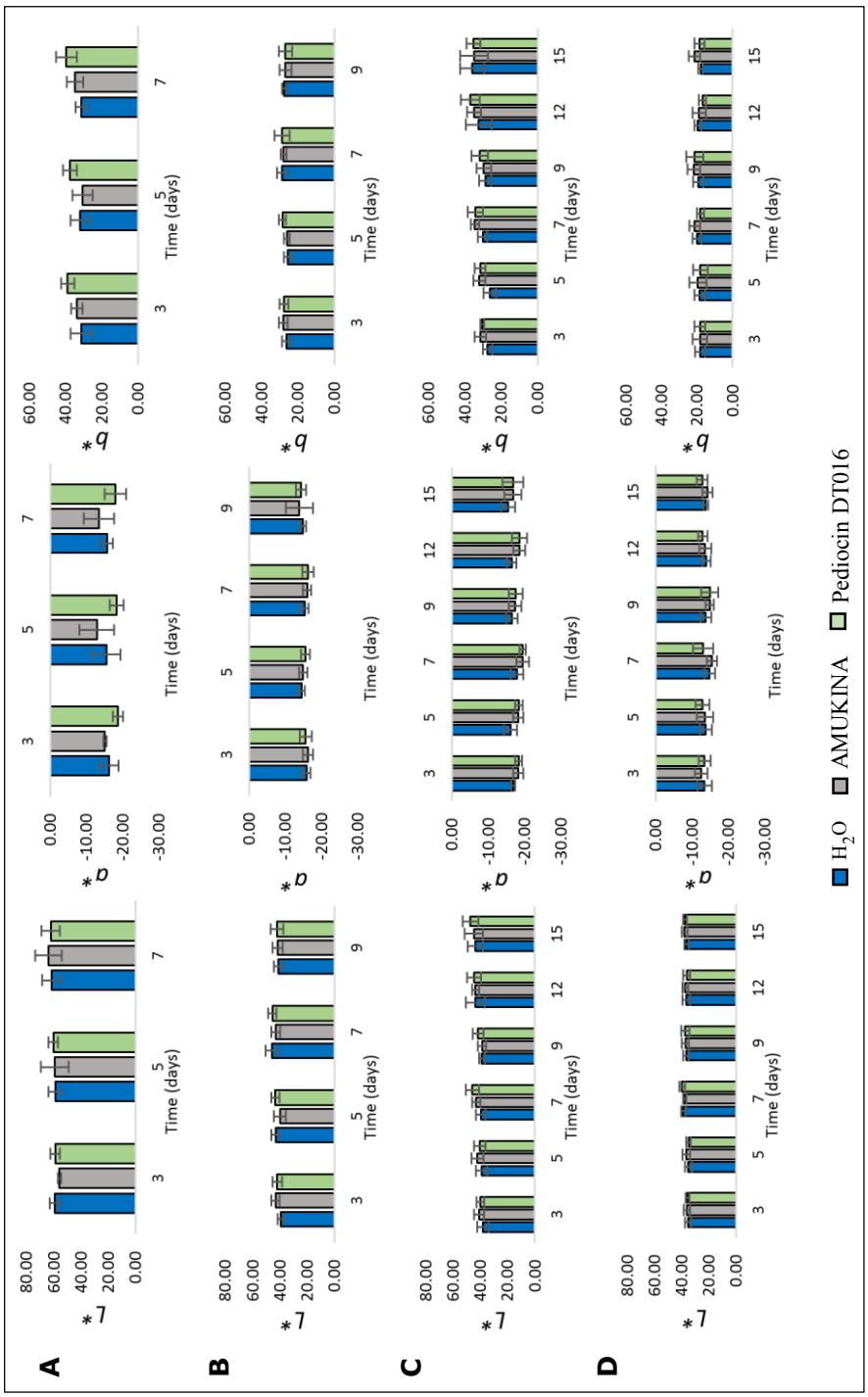

Figure 5. Colour coordinates of fresh vegetables washed with water, AMUKINA and pediocin DT016 solution, along storage at 4 ºC. The vegetables are A: Lettuce; B: Rocket salad; C: Parsley; D: Spinach. Error bars show standard deviation (SD).

5.5. Conclusions

5.5.1. Efficacy of Pediococcus pentosaceus DT016 against Listeria monocytogenes populations inoculated in fresh vegetables

At refrigeration temperature, *L. monocytogenes* was able to survive and grow in all the fresh vegetables. The addition of *P. pentosaceus* DT016 to the vegetables reduced and inhibited the pathogen populations, independently of the pathogenic load. The fresh vegetables showed to be a suitable environment for the protective culture survival, growth and bacteriocin production. The addition of *P. pentosaceus* did not influence the colour parameters of lettuce, rocket salad and parsley. Only after prolonged storage, the spinach samples with the protective culture showed different a^* and b^* properties.
In conclusion, *P. pentosaceus* DT016 can be effective as a protective culture for fresh vegetables kept under cold storage.

5.5.2. Effect of washing solutions on Listeria monocytogenes populations inoculated in fresh vegetables

The washing procedure of the vegetables with pediocin DT016 solution significantly reduced the initial load of *L. monocytogenes* and inhibited the pathogen proliferation during prolonged storage at 4 ºC. The pathogen was able to survive and grow in the vegetables washed with water and AMUKINA, during the storage at 4 ºC.
The pediocin washing did not affect the colour properties of lettuce, rocket salad and spinach. Although the parsley samples washed with the pediocin presented differences in green colour compared with the samples washed with water, at the end of storage all the colour parameters were similar between these washings.
The application of washing solutions containing pediocin DT016 is a very promising method to inhibit *L. monocytogenes* proliferation in fresh vegetables. Combined

treatments with other inhibitory agents or sanitizers and the use of more concentrated bacteriocin solutions should be considered to completely eradicate the pathogen.

5.6. Acknowledgements

This work was supported by National Funds from FCT – Fundação para a Ciência e a Tecnologia through project PEst-OE/EQB/LA0016/2013.
Financial support for authors Ramos B. and Brandão T.R.S. was provided by FCT and Fundo Social Europeu (FSE) through fellowships SFRH/BD/42169/2007 and SFRH/BPD/41419/2007, respectively.

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Chapter 6

General conclusions and Suggestions for further work

6.1. General Conclusions

This study focused on the control of the foodborne pathogen *Listeria monocytogenes* in fresh vegetables using natural alternatives.

The gathered literature, presented in Chapters 1 and 2, may be used in the development of effective alternative approaches for human pathogens´ control in fresh and minimally processed vegetables.

In Chapter 3, the use of various vinegar solutions to improve the safety of fresh vegetables consumption, in home and retail environments, was evaluated. The results showed the beneficial effects of natural antimicrobials present in vinegar solutions to improve safety of minimally processed lettuce. Balsamic vinegar showed similar and even better effectiveness than chlorine-based sanitizers in removing *Listeria monocytogenes* from lettuce surface, even though the increased storage time of inoculated lettuce allowed the bacterial attachment. In addition, the results revealed that the Balsamic vinegar may be an effective washing solution to inhibit food pathogens from produce, in households and food establishments.

Chapter 4 considers the promising possibility of using autochthonous LAB from vegetables in strategies of biopreservation. Strain DT016, previously from isolated Iceberg lettuce, with antagonism towards *L. monocytogenes, Listeria innocua* and *Enterococcus faecalis* due to the production of a bacteriocin, was studied. The bacteriocinogenic strain was identified as *Pediococcus pentosaceus* by 16S rRNA sequencing (similarity of 99%). The bacteriocin produced by this strain was between 11 and 17 kDa in size, with bacteriostatic activity against *Listeria* cells and showed homology with pediocin PA-1. The antagonistic activity against *L. monocytogenes* and

L. innocua was observed at different temperatures, being of particular interest the maintenance of bacteriocinogenic activity at 4 ºC. In addition, pediocin DT016 was stable in a wide range of pH and temperature values.
For the first time a *Pediococcus pentosaceus* strain, which produces a bacteriocin similar to pediocin PA-1/AcH, was isolated from fresh lettuce with potential to control *L. monocytogenes* in fresh and minimally processed produce.

In Chapter 5, regarding the introduction of biocontrol agents in fresh and minimally processed produce, the use of the strain *Pediococcus pentosaceus* DT016 in fresh lettuce, rocket salad, parsley and spinach provided encouraging results concerning their safety and shelf life. The addition of the protective culture resulted on a reduction of the pathogen in all the vegetables. At the end of storage, a difference in *L. monocytogenes* population by a minimum of 1.4 log units was observed between the vegetables with *P. pentosaceus* and the vegetables without the protective culture.
The results also highlighted the importance of isolation and selection of biocontrol agents from commercial products of the same type, and the ability of the strain to colonize the product and survive under stringent conditions of refrigerated storage. In fact, *P. pentosaceus* DT016 was able to survive and maintain levels above 6.5 log CFU/g in all the vegetables at 4 ºC. Although, no noticeable changes in the colorimeter analyses were observed between the samples of the other vegetables ($p>0.01$), after prolonged storage the samples of spinach presented an increase in green colour coupled with an increase in yellow aspect.

Also in Chapter 5, the use of pediocin DT016, i.e. the bacteriocin produced by *P. pentosaceus* DT016, as a biocontrol agent applied during a washing step showed enhanced potential to increase the safety of fresh and minimally processed vegetables. Washing with pediocin DT016 reduced and prevented *Listeria* proliferation in all the vegetables. For long storage, the pathogen reductions achieved with the pediocin were always higher by at least 2.5 and 1.9 log CFU/g than the achieved with water and AMUKINA ($p<0.01$), respectively.

Other promising result is the fact that treatment with the pediocin DT016 did not significantly affect survival and proliferation of the non-*Listeria* populations present in all the vegetables. Natural microbiota present in fresh vegetables can play an important role in maintaining their quality and safety. In this respect, this can be a competitive advantage.
Significant changes in the colour parameters studied were not observed in all the vegetables treated with pediocin DT016 ($p>0.01$).

Therefore, regarding Chapter 5, the selected biocontrol agents may represent a good strategy to increase the safety and the shelf-life of fresh and minimally processed fruits and vegetables. Furthermore, since important health properties have been attributed to lactic acid bacteria, their use could also contribute to confer specific healthy properties to these products. However, the introduction of the biocontrol agents can be further optimized, focusing on the level and mode of inoculation and to limit the possible negative effects observed in the colour and sensorial parameters. In addition, the production of pediocin DT016 by the LAB culture needs to be optimize in order to be a feasible approach.

6.2. Suggestions for Further Work

The results obtained in this thesis allowed to bring with new research ideas, such as:

- To study the use of balsamic vinegar solutions on the control of other foodborne pathogens and their application in other food matrices, like fruits and other vegetables, and on the possible effect in the produce sensorial characteristics;
- To study the phenolic compounds naturally presented in balsamic vinegar, grape and grape juices for their antilisterial activity and future application as natural food preservatives;

- To analyse the overall quality, sensory acceptability and the shelf-life of fresh vegetables treated with the protective culture *Pediococcus pentosaceus* DT016 and the pediocin produced;
- To study possible applications of *P. pentosaceus* DT016 and pediocin DT016 as biopreservative agents in other food systems, like fruits and other vegetables;
- To optimize the application of *P. pentosaceus* DT016 and production of the pediocin DT016 in order to scale up their application in food processing, and for the marketing of the biopreservation agents;
- To study the combination of the biopreservation agents, *P. pentosaceus* DT016 and pediocin DT016, in the biocontrol of *L. monocytogenes* in minimally processed vegetables;
- To test synergistic combinations of the biopreservation agents described with other bacteriocins and mixed culture bacteriocinogenic LAB, towards pathogen biocontrol in vegetable and fruit systems;
- To combine the application of the biopreservatives agents with other preservative technologies as post-harvest intervention for the fresh vegetables industry, under the concept of hurdle technologies;
- To evaluate the influence of these agents on the microbiota of the produce and the gut microbiota;
- To study the virulence of surviving *L. monocytogenes* cells to these treatments*;*
- To assess the potential of Pediococcus *pentosaceus* DT016 as a probiotic strain.