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Estudo e controlo dos principais patogénios de origem alimentar em fruta minimamente processada

Doutoramento em Ciências Agrárias

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Declaração de autoria de trabalho

Declaro ser a autora deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

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Resumo

Os objetivos do presente trabalho foram estudar a qualidade microbiológica de frutas minimamente processadas (FMP), o crescimento de microrganismos patogénicos em pera 'Rocha' e o efeito da iluminação Ultravioleta-C (UV-C) e água electrolisada ácida e neutra (AE), enquanto métodos de desinfeção, de maçã 'Golden' e pera 'Rocha'. As populações de microrganismos aeróbios mesófilos e psicrotróficos, bactérias ácido-lácticas (BAL), coliformes, fungos, *Staphylococcus aureus*, *Salmonella* spp. e *Listeria monocytogenes* foram estudadas em 240 amostras de FMP (maçã, abacaxi, manga, papaia, melão, melão Cantaloupe, melão Gália e melancia). O número de microrganismos mesófilos e psicrotróficos variou de 3,0-9,2 e 2,2-10,7 log ufc/g, respetivamente, de BAL de 1,9-9,0 log ufc/g e de fungos de 2,3-10,4 log ufc/g. *Escherichia coli*, *Salmonella* spp. e *L. monocytogenes* nunca foram detetadas mas os coliformes estiveram presentes na totalidade das amostras. *E. coli*, *Salmonella enterica* e *Listeria* spp. cresceram em pera com taxas específicas de crescimento elevadas a 12°C (1,94-2,6 dia^{-1}) e a 20°C (2,68-3,08 dia^{-1}), atingindo populações microbianas de 8,1-8,6 log ufc/g, em 24 h. A 4°C observou-se crescimento exponencial após fases de adaptação inferiores a 24 h para *Listeria* spp. e a 8 dias para as enterobactérias. A iluminação UV-C revelou-se um método muito eficaz de descontaminação de FMP tendo causado reduções de 1,0-3,4 log ufc/g nas populações de *E. coli*, *S. enterica* e *Listeria* spp. em maçãs e peras minimamente processadas enquanto que a AE resultou em reduções semelhantes às obtidas com hipoclorito de sódio (≈1 log ufc/g) tradicionalmente utilizado na indústria. A dose de UV-C 7,5 kJ/m^2, foi a que originou reduções microbianas maiores. Nenhum dos métodos de descontaminação utilizados produziu alterações significativas da qualidade das FMP. Os resultados alertam para a importância da prevenção da contaminação, da aplicação de métodos de descontaminação eficazes e do controlo das condições de refrigeração durante o processamento e comercialização de FMP.

Palavras chave: qualidade microbiológica de fruta minimamente processada, *Escherichia coli*, *Listeria monocytogenes*, *Salmonella enterica*, água electrolisada, Iluminação Ultravioleta-C

Abstract

The objectives of this study were to investigate the microbiological quality of minimally processed fruits (MPF), the growth of foodborne pathogens in 'Rocha' pear and the effect of Ultraviolet-C (UV-C) illumination and electrolyzed water (EW), acidic and neutral, as disinfection methods of 'Golden' apples and 'Rocha' pears. Mesophilic and psychrotrophic aerobic microorganisms, lactic-acid bacteria (LAB), coliforms, fungi, *Staphylococcus aureus*, *Salmonella* spp. and *Listeria monocytogenes* were studied in 240 samples of MPF (apple, pineapple, mango, papaya, melon, Cantaloupe melon, Galia melon and watermelon). The number of mesophilic and psychrotrophic microorganisms ranged from 3.0-9.2 and from 2.2-10.7 log cfu/g, respectively, of BAL from 1.9-9.0 log cfu/g and of fungal from 2.3-10.4 log cfu/g. *Escherichia coli*, *Salmonella* spp. and *L. monocytogenes* were not detected, although the coliforms were found in all the fruit samples. *E. coli*, *Salmonella enterica* and *Listeria* spp. grew in pear with high specific growth rates at 12°C (1.94-2.6 day^{-1}) and 20°C (2.68-3.08 day^{-1}), reaching microbial populations of 8.1-8.6 log cfu/g in 24 h. At 4°C exponential growth was observed after adaptation phases less than 24 h for *Listeria* spp. and 8 days for enterobacteria. UV-C light appeared to be the most effective method of decontamination of MPF having caused microbial reductions from 1.0 to 3.4 log cfu/g in the populations of *E. coli*, *S. enterica* and *Listeria* spp. on minimally processed apples and pears, while the AE resulted in microbial reductions similar to those obtained with sodium hypochlorite (≈1 log cfu/g), traditionally used in the industry. In general, the UV-C dose of 7.5 kJ/m^2 caused the highest microbial reductions. None of the disinfection methods used resulted in significant alterations in the quality of MPF. The results highlight the importance of preventing contamination, selecting an adequate decontamination technology and maintaining a strict temperature control during processing, distribution and selling of minimally processed fruits.

Keywords: Fresh-cut fruit microbiological quality, *Escherichia coli*, *Listeria monocytogenes*, *Salmonella enterica*, electrolyzed water, Ultraviolet-C illumination

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Lista de abreviaturas e acrónimos

- Lista de abreviaturas e acrónimos em português

ADN – Ácido desoxirribonucleico
AE – Água electrolisada
AEA – Água electrolisada ácida
AEN – Água electrolisada neutra
ATP – Trifosfato de adenosina
a_w – Atividade da água
BAL – Bactérias ácido-lácticas
CE – Comissão Europeia
CECT – Colección española de cultivos tipo
CK – controlo
cv – Cultivar
DEAC – *Escherichia coli* de aderência difusa
EAEC – *E. coli* enteroagregativa
EIEC – *E. coli* enteroinvasora
EHEC – *E. coli* enterohemorrágica
EPEC – *E. coli* enteropatogénica
ETEC – *E. coli* enterotoxigénica
FMP – Frutas minimamente processadas
VIH – Vírus da Imunodeficiência Humana
HUS – Síndrome urémico hemolítico
INE – Instituto Nacional de Estatística
OMS – Organização Mundial de Saúde
PMP – Produtos Minimamente Processados
STEC –*E. coli* produtora de toxina Shiga
ufc – Unidades formadoras de colónias
UV – Ultravioleta
UV-A – Ultravioleta-A
UV-B – Ultravioleta-B
UV-C – Ultravioleta-C
VTEC –*E. coli* produtora deVerocitoxina

- Lista de abreviaturas e acrónimos em inglês

AC – After cutting
AEW – Acidic electrolyzed water
AM – Aerobic mesophilic microrganisms
AP – Aerobic psychrotrophic microrganisms
ATCC – American type culture collection
ATR – Acid tolerance response
bp – base pair
BOPP – Biaxially-oriented polypropylene
BPW – Buffered peptone water
CDC – Center for Disease Control and Prevention
cfu – Colony-forming unit
CSPI – Center for Science in the Public Interest

DNA – Deoxyribonucleic acid
DW – Distilled water
EFSA – European Food Safety Authority
EW – Electrolyzed water
FAO – Food and Agricultural Organization
FCF – Fresh-cut fruit
FDA – Food and Drug Administration
HGT – Horizontal gene transfer
HPP – High Pressure Processing
ICMSF – International Comission on Microbiological Specifications for Foods
IFPA – International Fresh-cut Produce Association
ISO – International Organization for Standardization
LAB – Lactic acid bacteria
MAP – Modified atmosphere packaging
MPF – Minimally processed fruits
NEW – Neutral electrolyzed water
NCTC – National collection of type cultures
ORP – Oxidation-reduction power
PCR – Polimerase chain reaction
PDO – Protected denomination of origin
PHAC – Public Health Agency of Canada
RFLP – Restriction fragment lenght polyphormism
SH – Sodium hypochlorite
SSC – solid soluble contents
TAE – Tris-acetate EDTA
TC – Total coliforms
TSA - Tryptic Soy Agar
TSB – Tryptic Soy Broth
TTA – titratable acidity
WHO – World Health Organization
YM – Yeast and molds

Plano geral da tese

A presente tese é constituída por 6 capítulos sendo o primeiro uma revisão bibliográfica geral, 4 capítulos organizados em artigos científicos (2 publicados e 2 submetidos) onde se descreve o trabalho de investigação realizado e um capítulo de considerações finais, conclusões e perspectivas futuras (Capítulo VI).

No primeiro capítulo (Capítulo I) apresenta-se o contexto e âmbito dos temas em estudo, enquadrados no conhecimento científico existente, resultante de pesquisa bibliográfica. Procurou-se dar ênfase aos aspetos que contribuíssem para o melhor enquadramento dos objetivos e compreensão das estratégias selecionadas para atingir esses objetivos.

O Capítulo II corresponde ao artigo intitulado "Evaluation of microbial quality and yeast diversity in fresh-cut apple" publicado no jornal "Food Microbiology", que reporta a qualidade microbiológica da maçã minimamente processada comercializada no mercado do Algarve, em diferentes tipos de comércio. Para tal, estudaram-se os parâmetros de qualidade microbiológica (microrganismos aeróbios mesófilos e psicrotróficos, bactérias ácido-lácticas, fungos filamentosos e leveduras e coliformes totais) e de segurança alimentar (*Escherichia coli, Salmonella* spp. e *Listeria monocytogenes*). Tendo em conta a importância da população de leveduras na maçã identificou-se uma amostra significativa dos isolados obtidos.

O Capítulo III integra o manuscrito "Study of the microbiological safety of minimally processed fruits commercialized in the South of Portugal", cujo objetivo foi estudar a qualidade microbiológica de fruta minimamente processada comercializada no Algarve. Estudaram-se os parâmetros de qualidade microbiológica e de segurança alimentar referidos no Capítulo II num conjunto de amostras de fruta (abacaxi, manga, papaia, melão, melão Cantaloupe, melão Gália, melancia, morangos e saladas de frutas). Determinou-se também o nível de alguns grupos microbianos em amostras de fruta armazenada a temperaturas de refrigeração depois do prazo de validade ter sido ultrapassado e compararam-se os valores obtidos com os de fruta estudada antes de terminado esse limite.

No Capítulo IV foi estudado o efeito de iluminação UV-C na inativação de *E. coli, Salmonella enterica* e *L. innocua in vitro* e em maçã minimamente processada. Os resultados deste estudo foram publicados no artigo "Low dose UV-C illumination as an eco-innovative disinfection system on minimally processed apples” no jornal “Postharvest Biology and Technology”.

No Capítulo V avaliou-se a capacidade de sobrevivência e crescimento de *E. coli*, *S. enterica* e *Listeria* spp. em pera 'Rocha'. Estudou-se também o efeito da iluminação UV-C e da água electrolisada ácida e da água electrolisada neutra, como métodos de desinfeção da pera minimamente processada e contaminada. Os resultados estão descritos no manuscrito “Growth of *Escherichia coli*, *Salmonella enterica* and *Listeria* spp., and their inactivation using ultraviolet energy and electrolyzed water, on fresh-cut 'Rocha' pear” que se encontra submetido.

Os estudos apresentados nesta dissertação para obtenção do grau de Doutor em Ciências Agrárias, com especialidade em Tecnologia de Alimentos, resultaram do trabalho de investigação desenvolvido entre 2011 e 2015, na Universidade do Algarve. Estes estudos foram apoiados pela Fundação para a Ciência e Tecnologia, através da bolsa de doutoramento com a referência SFRH/BD/76745/2011. O trabalho experimental foi realizado no âmbito do projeto de investigação “A novel approach to control pathogen contamination and enhance safety and quality on fresh-cut fruit” (SafeFCF) PTDC/AGR-ALI/111687/2009.

Os resultados descritos nesta dissertação, além de publicados em revistas internacionais, também foram divulgados em congressos científicos nacionais e internacionais sob a forma de comunicações orais e em painel.

Capítulo I

Introdução

1. Introdução

Em 2004, um painel de peritos da Organização das Nações Unidas para a Alimentação e a Agricultura (FAO) e da Organização Mundial de Saúde (OMS) recomendou um consumo mínimo diário de 400 g de frutas ou vegetais frescos para a prevenção da deficiência de micronutrientes e de doenças crónicas. Os frutos e os vegetais são constituintes essenciais de uma dieta saudável pois representam uma fonte de vitaminas e outros nutrientes, cuja ingestão poderá diminuir o risco de desenvolvimento de doenças cardiovasculares, diabetes e certos tipos de cancro. Está estimado que um aumento do consumo de fruta e vegetais poderá contribuir para a prevenção de 2,7 milhões de mortes anualmente (FAO/WHO, 2005).

Os dados estatísticos referentes ao consumo de fruta em Portugal permitiram concluir que houve uma diminuição de 9,5 % entre 2008 e 2012 (INE, 2014). Esta diminuição pode ser devida ao facto de, atualmente, muitas refeições serem feitas fora de casa, sendo mais fácil optar por "comida rápida" o que pode tornar o acesso a alimentos de origem vegetal frescos mais difícil. Os produtos minimamente processados (PMP), nomeadamente as frutas minimamente processadas (FMP), prontos a ingerir poderão ser uma boa alternativa para o consumo de fruta fresca fora de casa.

A International Fresh-Cut Produce Association (IFPA) define PMP como frutas ou vegetais que sofreram alterações físicas mas mantêm o seu estado fresco e as propriedades naturais dos alimentos inteiros dos quais derivaram. Estes alimentos, também designados como produtos de IV gama, são cortados e/ou descascados em produtos 100 % utilizáveis, embalados, e oferecem aos consumidores um alto valor nutritivo, conveniência e sabor (IFPA, 2002). As frutas e os vegetais minimamente processados são alimentos que se encontram crus, prontos a consumir ou cozinhar não sendo submetidos a qualquer tipo de processamento térmico (Oliveira *et al.*, 2015). Estes alimentos devem ser armazenados, distribuídos e comercializados em condições de refrigeração. O seu tempo de vida útil ou tempo de prateleira difere consoante o produto, podendo variar entre 7 e 20 dias se for mantido à temperatura recomendada (≤4 °C) (Watada e Qi, 1999).

A indústria de PMP surgiu nos Estados Unidos da América (EUA) para abastecer hotéis, restaurantes, serviços de *catering* e outras instituições. Estes alimentos estão disponíveis nos supermercados desde os anos 40 do século XX. Na Europa, os vegetais minimamente processados surgiram no início da década de 80 em França (Rojas-Grau *et al.*, 2011). O Reino Unido possui, atualmente, o maior mercado de PMP da União Europeia, sendo responsável por cerca de um terço do consumo total deste tipo de alimentos na UE (Oliveira *et al.*, 2015).

1.1. Produção de fruta minimamente processada

O processamento de FMP comporta numa série de etapas que incluem operações de seleção, lavagem, descasque, corte/redução de dimensões, descontaminação/desinfeção, embalagem e armazenamento as quais devem ocorrer em ambiente refrigerado (Figura 1.1) (Corbo *et al.*, 2010). Cada uma das operações deve ser efetuada de maneira a assegurar que a qualidade, o tempo de vida útil e a segurança microbiológica do produto final sejam as máximas possíveis (Gorny, 1996; Oliveira *et al.*, 2015). De forma a obter-se um PMP microbiologicamente seguro e com propriedades nutricionais elevadas deve acautelar-se a qualidade da matéria-prima (boas condições de cultivo, colheita e armazenamento) e a qualidade da água de lavagem (água potável) (FAO, 2011; Olaimat e Holley, 2012).

1.1.1. Seleção e lavagem

Numa primeira fase, procede-se a uma seleção dos frutos onde são separados os frutos sãos e sem defeitos e são efetuadas análises de qualidade (cor, acidez, °Brix, entre outros). A qualidade de um alimento minimamente processado está relacionada com a qualidade do produto inteiro (Watada *et al.*, 1996). O fruto inteiro tem de suportar as diferentes operações unitárias as quais podem provocar stresse resultando num aumento da taxa de respiração e emissão de etileno, acelerando a sua degradação (Soliva-Fortuny e Martín-Belloso, 2003). Os frutos num estado de maturação avançado não são adequados ao processamento mínimo pois limitam o seu tempo de vida útil. Por outro lado, os frutos num estado de maturação precoce também não são apropriados devido às suas características sensoriais (Watada e Qi, 1999).

A etapa seguinte é a lavagem dos frutos de modo a remover a sujidade proveniente do campo (resíduos de pesticidas, insetos, entre outros). Após a lavagem, os frutos devem ser refrigerados de imediato para baixar a sua temperatura interna atrasando assim os processos biológicos que levam ao seu amadurecimento (Artés e Allende, 2014).

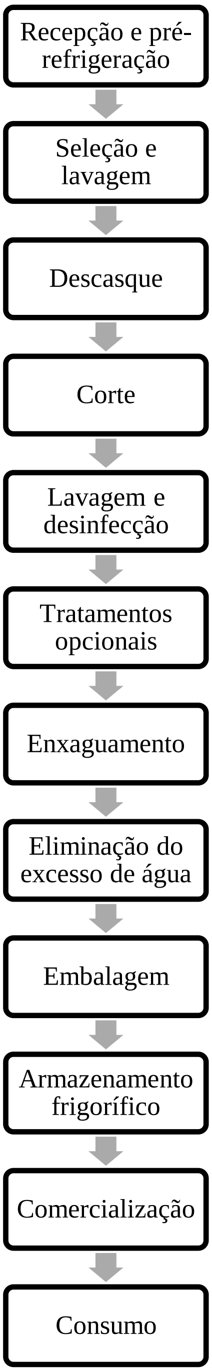

Fig. 1.1. Etapas do processamento industrial de fruta minimamente processada (Corbo *et al.*, 2010; Garcia e Barret, 2005) .

1.1.2. Descasque e corte

O descasque permite a eliminação da camada externa nos casos em que esta não é comestível ou quando a apresentação final do alimento assim o exige. Esta operação pode ser manual, mecânica (descascador mecânico) ou enzimática. Pode também realizar-se recorrendo a água quente ou vapor sob alta pressão (Artés e Allende, 2014). A operação de descasque contribui para a redução da carga microbiana presente na matéria-prima. Contudo, como são removidas as barreiras exteriores, o alimento fica mais suscetível a contaminações microbianas que ocorram após o processamento mínimo, resultantes de contaminações cruzadas ou devidas a um manuseamento inadequado (Oms-Oliu *et al.*, 2010). Esta operação deve ser realizada da forma menos abrasiva possível de modo a prevenir a invasão dos tecidos por microrganismos e evitar escurecimentos internos (Garcia e Barret, 2005; Corbo *et al.*, 2010).

O corte é uma operação de redução de dimensões através da qual o produto adquire o seu aspeto final. O alimento pode ser fatiado, picado, ralado, cortado em cubos ou em secções e estas operações podem ser manuais ou mecânicas. A dimensão do produto final dependerá do uso ao qual está destinado (FAO, 2011). A utilização de utensílios de corte afiados reduz os danos físicos fazendo com que o stresse das células seja menor (Garcia e Barret, 2005; FAO, 2011). Esta operação deve ser efetuada com o máximo cuidado higiénico e todos os utensílios devem estar limpos e desinfetados de maneira a minimizar potenciais contaminações microbianas (FAO, 2011; Oms-Oliu *et al.*, 2010).

1.1.3. Lavagem e desinfeção

As operações de lavagem e desinfeção são realizadas, normalmente, em simultâneo. Para tal, adicionam-se agentes antimicrobianos à água onde decorrem as lavagens sendo esta a única etapa de redução da carga microbiana existente durante o processamento dos PMP/FMP (Corbo *et al.*, 2010; Gil *et al.*, 2009).

A lavagem pode ser feita por pulverização com água, embora geralmente envolva a imersão do fruto, durante um período de tempo pré-estabelecido, em água refrigerada (1–10 °C), em tanques que contêm uma concentração entre 50 e 200 ppm de cloro livre, acidificada com ácido cítrico (150-200 ppm) de maneira a manter o valor do pH entre 6,5 e 7,5 para se obter uma atividade antimicrobiana elevada (FAO, 2004). A qualidade microbiológica e química da água de lavagem deve ser monitorizada de forma a evitar contaminações (Gil *et al.*, 2009). Nesta fase, pode-se adicionar agentes anti-oxidantes para prevenir o escurecimento dos frutos (Artés e Allende, 2014). Assim, os principais objetivos destas operações são a diminuição da temperatura dos frutos, a eliminação da sujidade e a diminuição da carga microbiana e dos fluídos dos tecidos (Garcia e Barret, 2005; Oms-Oliu *et al.*, 2010).

1.1.4. Enxaguamento e eliminação do excesso de água superficial

Depois da lavagem, procede-se ao enxaguamento com o objetivo de remover os possíveis resíduos dos desinfetantes dos alimentos minimamente processados obtidos.

A humidade superficial residual e o exsudado na superfície dos recém-cortados frutos podem estimular o crescimento de microrganismos como fungos e bactérias (FAO, 2011). A eliminação do excesso de água superficial deve ser efetuada de forma cuidadosa para evitar danos nos tecidos dos frutos, podendo ser realizada em transportadores rotativos, grelhas vibratórias, túneis de secagem ou por ar forçado, durante um período de tempo adequado ao produto (Artés e Allende, 2014; Soliva-Fortuny e Martín-Belloso, 2003).

1.1.5. Embalamento

O embalamento é uma etapa crítica no processamento de PMP/FMP e deve ser efetuado com a máxima higiene. Nesta fase, o produto é pesado e colocado na embalagem, podendo também ser realizadas misturas de vários frutos, no caso das misturas de frutas.

Dependendo dos alimentos, o acondicionamento pode ser realizado em bolsas, em caixas ou em bandejas e podem ser usados diferentes tipos de filmes protetores (FAO, 2011). Pode-se optar por embalar o alimento em atmosfera modificada (MAP), ou seja, modificar a composição de gases dentro da embalagem com o objetivo de prolongar o tempo de vida útil dos produtos (Oliveira *et al.*, 2015), minimizando a atividade metabólica, atrasando o escurecimento enzimático e mantendo a aparência do alimento, através da redução da taxa de respiração e da produção de etileno (Ramos *et al.*, 2013). Atmosferas pobres em O_2 potenciam o metabolismo anaeróbico dos frutos minimamente processados, o que leva ao aumento da fermentação (Corbo *et al.*, 2010) podendo causar a formação de acetaldeído e compostos que alteram o sabor (Rico *et al.*, 2007); as concentrações elevadas de CO_2 inibem uma série de enzimas do ciclo de Krebs (Soliva-Fortuny e Martín-Belloso, 2003). No que diz respeito ao crescimento microbiano, atmosferas com concentrações baixas de O_2 inibem o crescimento da maioria dos microrganismos degradadores aeróbios como as bactérias Gram negativas (ex: *Pseudomonas*). Contudo, o crescimento de microrganismos patogénicos, psicrotróficos anaeróbios e clostridios não proteolíticos, pode ser estimulado (Al-Ati e Hotchkiss, 2002; Soliva-Fortuny e Martín-Belloso, 2003).

Após o embalamento, procede-se a uma inspeção visual da qualidade e colhem-se amostras para a realização de ensaios de qualidade (microbiológicos e químicos). O produto passa depois por um controlo de peso e por detetores de metais para eliminar o risco de presença deste tipo de elementos (FAO, 2011). As embalagens são posteriormente colocadas em caixas de cartão e armazenadas a temperaturas de refrigeração antes de serem expedidas. A temperatura é um dos fatores mais importantes na sobrevivência e crescimento dos microrganismos nos frutos minimamente processados. Esta deve ser controlada durante todo o processamento de modo a assegurar a qualidade e segurança do alimento e a temperatura de armazenamento deve ser de 4 °C ou inferior de modo a prevenir o crescimento dos microrganismos degradadores e patogénicos (Oliveira *et al.*, 2015).

1.2. Métodos de desinfeção

Durante o processamento de PMP/FMP, as etapas de lavagem e desinfeção utilizando substâncias antimicrobianas são as únicas que poderão permitir uma redução das populações de microrganismos de degradação e de microrganismos patogénicos (Allende *et al.*, 2006; Corbo *et al.*, 2010). A desinfeção é um dos passos fundamentais do processamento mínimo de alimentos de origem vegetal pois afeta a qualidade, a segurança e o tempo de prateleira do produto final (Gil *et al.*, 2009).

Existem vários métodos para reduzir a carga microbiana superficial das frutas e vegetais embora o ideal seja sempre prevenir a contaminação dos mesmos (Ramos *et al.*, 2013). No entanto, nem sempre é possível evitar as contaminações sendo necessário o recurso a técnicas que reduzam/eliminem os microrganismos patogénicos e de degradação de modo a evitar doenças de origem alimentar e perdas económicas (Corbo *et al.*, 2010).

Os métodos utilizados na desinfeção/descontaminção da fruta baseiam-se em processos físicos, químicos ou na combinação dos dois. Contudo, o cloro é o agente desinfetante mais utilizado na indústria de processamento de alimentos frescos minimamente processados (Gil *et al*, 2009; Goodburn e Wallace, 2013; Olaimat e Holley, 2012; Rico *et al.*, 2007).

1.2.1. Cloro

Na indústria de processamento de PMP/FMP, o cloro é normalmente usado sob a forma de soluções de hipoclorito de sódio (NaClO) devido à sua eficiência, fácil manuseamento e baixo custo. A sua aplicação é realizada em concentrações que variam entre 50 e 200 ppm durante um tempo de exposição de 1-2 min (Beuchat, 1998) e a sua atividade inibitória ou letal depende da concentração de cloro disponível para reações de oxidação e desinfeção, do tempo de contacto e do pH (Luo *et al*., 2011a; Suslow, 1997). Em solução aquosa, os sais de hipoclorito formam uma mistura de ácido hipocloroso (HOCl), cloro gasoso (Cl_2) e iões hipoclorito (OCl^-), em quantidades que variam dependendo do pH e da temperatura da solução (Beuchat, 1998; Gil *et al*., 2016; Suslow, 1997). Diversos estudos têm mostrado que o HOCl, que é um ácido fraco, é a forma com maior atividade microbicida pois é a forma que se difunde para o interior das células (Beuchat, 1998; Zagory, 2000). Tendo em conta que em solução a quantidade das diferentes formas de cloro depende do pH, verificou-se que a gama de valores de pH que garantem um melhor compromisso de estabilidade e atividade microbicida do HOCl se situa entre 6,5 e 7,5 (Izumi, 1999; Suslow, 1997). Valores de pH mais baixos favorecem a formação de cloro na forma gasosa que é libertado para o ambiente podendo provocar intoxicações nos operadores (Izumi, 1999; Suslow, 1997; Zagory, 2000). Valores de pH mais altos favorecem a reação do cloro com compostos azotados produzindo cloraminas (Suslow, 1997) e a dissociação de HOCl em iões de OCl^- (Izumi, 1999). Estas reações, para além de originarem substâncias tóxicas, contribuem para a diminuição do efeito biocida do cloro (Ramos *et al*., 2013; Zagory, 2000).

O modo de atuação do HOCl sobre os microrganismos ainda não está bem elucidado podendo ser resultado de uma série de aspetos, tais como: a oxidação de aminoácidos e de enzimas; a perda de constituintes intracelulares; a interferência nos mecanismos de transporte de nutrientes e de oxigénio; a inibição da síntese de proteínas; a diminuição da produção de ATP; a indução de quebras nas moléculas de ADN; e a inibição da síntese de ADN (Maris, 1995; CDC, 2008a). Os níveis de reduções bacterianas obtidos quando se utilizam soluções de hipoclorito de sódio nos alimentos minimamente processados, nas concentrações e tempos de contacto permitidos, são de cerca de 1 a 2 log ufc/g (Johnston *et al*., 2005; Parish *et al*., 2003).

A utilização do cloro como desinfetante está associada a riscos para o ambiente e para a saúde humana pois as reações químicas do cloro com diversos compostos orgânicos presentes na matriz alimentar podem originar compostos tóxicos, potencialmente cancerígenos e mutagénicos, como os trihalometanos, ácidos haloacéticos, halocetonas, cloropicrinas e as cloraminas (Allende *et al*., 2008; Deborde e Gunten, 2008; Escalona *et al*., 2010; Gil *et al*., 2009, 2016; Richardson *et al*., 2007). Consequentemente, em alguns países como a Alemanha, Holanda, Suíça e Bélgica o uso de cloro em alimentos minimamente processados está proibido (Rico *et al*., 2007).

1.2.2. Métodos de desinfeção alternativos ao cloro

Devido às limitações legais, de eficácia e ao risco associado do uso de cloro enquanto agente desinfetante na indústria de produção de vegetais e frutos minimamente processados, tornou-se imperioso o desenvolvimento de estratégias de descontaminação alternativas que permitam obter estes alimentos com boa qualidade sensorial e microbiológica e que, ao mesmo tempo, sejam seguros tanto para a saúde humana como para o ambiente.

Os principais métodos químicos que atualmente se apresentam como alternativos ao uso do cloro como desinfetante na indústria de alimentos minimamente processados são: o clorito de sódio acidificado, o peróxido de hidrogénio, o ácido peracético, a água electrolisada (água electrolisada ácida e água electrolisada neutra), o ozono, os ácidos orgânicos e os compostos alcalinos, cujas vantagens, limitações e aplicações se encontram resumidas na Tabela 1.1.

Os métodos de desinfeção físicos com maior potencial como substitutos do cloro, são, a iluminação UV-C, a irradiação (raios gama, raios X, feixe de electrões), a luz pulsada, a alta pressão e os ultrassons, cujas principais características se encontram sintetizadas na Tabela 1.2.

Tabela 1.1. Métodos químicos utilizados para reduzir a carga microbiana de frutas e vegetais.

Método	Vantangens e desvantagens	Aplicações/Eficácia	Referências
Cloro (Hipoclorito de sódio)	- Fácil manuseamento e baixo custo - Sub-produtos potencialmente prejudiciais para a saúde humana e para o ambiente - Eficácia reduzida na presença de matéria orgânica e dependente do pH da solução - Proibido em alguns países europeus	[50-200 ppm]; 1-2 min Redução: 1-2 log ufc/g	Allende *et al*., 2008 Beuchat, 1998 Gil *et al*., 2009 Parish *et al*., 2003 Ramos *et al*., 2013 Rico *et al*., 2007
Clorito de sódio acidificado	- Eficácia superior ao hipoclorito de sódio devido ao pH baixo (2,5-3,2) - Corrosivo - Pouca informação acerca da formação de sub-produtos clorados	[500 a 1200 ppm] Redução: 3 log ufc/g	FDA, 2012 Luo *et al.*, 2011b Parish *et al*., 2003 Park e Beuchat, 1999 Ramos *et al*., 2013
Peróxido de hidrogénio	- Não produz resíduos; decomposição em compostos não tóxicos e não é corrosiva - Esporicida - Pode causar escurecimento ou perda de cor do alimento - Não permitido em alimentos BIO; fitotóxico - Eficácia antimicrobiana reduzida nas concentrações permitidas em vegetais e eficácia reduzida contra fungos e vírus	[0,04 e 1,25%] Eficácia reduzida Redução de qualidade entre 4 a 5%,	FDA, 2013 Olmez *et al*., 2009 Parish *et al*., 2003 Park e Beuchat, 1999 Ramos *et al*., 2013
Ácido peracético	- Não produz sub-produtos prejudiciais e não é corrosivo à concentração autorizada - Eficácia não afetada pela matéria orgânica e temperatura; eficácia de desinfeção superior ao cloro - Aumento de matéria orgânica nos efluentes devido à presença de ácido acético - Aumento do potencial de crescimento dos microrganismos - Redução insuficiente da carga microbiana com a concentração máxima permitida	[max., 80 ppm]	FDA, 2019 Kitis, 2004 Ramos *et al*., 2013
Água electrolisada	AEN - Não afeta o pH, a cor e a aparência do alimento; não é corrosiva, menos irritante para a pele e mais estável à perda de cloro - Eficácia de desinfeção inferior à AEA AEA - Eficácia antimicrobiana superior ao cloro devido ao potencial de oxidação redução elevado - Corrosiva para os equipamentos devido ao pH baixo	[50-100 ppm]; 3-5 min Redução: 0,6-2,6 log ufc/g	Abadias *et al*., 2008 Graça *et al*., 2011 Izumi *et al*., 1999 Ramos *et al*., 2013 Rico *et al*., 2007
Ozono	- Eficaz a baixas concentrações e por curtos períodos de exposição e amplo espetro de ação e grande capacidade de penetração - Decomposição em produtos não tóxicos - Possibilidade de danos fisiológicos nos alimentos e corrosão e deterioração dos metais e superfícies - Instável e altamente reativo; exposições prolongadas a concentrações acima de 4 ppm podem ser letais para os humanos - Normalmente é usado para o tratamento de águas	- Aquoso: [0,03-20 ppm] - Gasoso: [máximo 20000 ppm]; 20 min-6 h	Beuchat, 1998 Horvath *et al*., 1985 Parish *et al*., 2003 Ramos *et al*., 2013
Ácidos orgânicos (láctico, cítrico, acético, tartárico ou ácido ascórbico)	- Económicos dependendo do tipo de ácido e do uso - Toxicidade reduzida; uso permitido em alimentos BIO - Efeito antimicrobiano dependente do tipo de ácido e do microrganismo - Possível efeito negativo na qualidade sensorial e impacto negativo nos efluentes	[0,5 e 5%]; 5-15 min Redução: 0,96-2,1	Chen *et al*., 2016 Ramos *et al*., 2013 Ramos-Villarroel *et al.*, 2015
Compostos alcalinos – Fosfato trissódico (FTS)	- Pouco corrosivo para os equipamentos - Problemas ambientais devido ao pH alto (11-12) e à presença de fosfatos - Eficácia reduzida contra *Listeria*	[1 e 15%] Redução: 0-6 log ufc/g	Parish *et al*., 2003 Ramos *et al*., 2013

Tabela 1.2. Métodos físicos utilizados para reduzir a carga microbiana de frutas e vegetais.

Método	Vantangens e desvantagens	Aplicação/Eficácia	Referências
UV-C	- Fácil manuseamento; custos reduzidos do equipamento, energia e manutenção - Ausência de resíduos - Estimulação de mecanismos de defesa na fruta que atrasa a senescência e degradação; aumento da atividade antioxidante da fruta e indução da síntese de compostos benéficos à saúde como antocianinas e estilbenos - Poder de penetração nos alimentos reduzido; composição do alimento afeta a eficácia - Possível indução de stresse na fruta, com aumento da respiração, alteração na cor e de *off-flavors*	[0,5-20 kJ/m^2] Redução: 1 a >2 log ufc/g	Bintsis *et al.*, 2000 Escalona *et al.*, 2010 George *et al.*, 2015 Manzocco *et al.*, 2011 Ramos *et al.*, 2013
Irradiação	- Pode ser efetuada à temperatura ambiente; pode ser aplicada após o embalamento - Atrasa o amadurecimento e a senescência de frutos climatéricos - Baixos custos energéticos - Não aceitação por parte dos consumidores - Possível perda de qualidade quando são aplicadas doses elevadas - Possíveis alterações na textura do alimento - Alta eficácia na eliminação de bactérias patogénicas e de parasitas da superfície de frutos e vegetais - Equipamento caro	[máximo de 1,0 kGy] Redução: 1,1-2,35 log ufc/g	Lacroix e Outtara, 2000 Lacroix, 2014 Palekar *et al.*, 2015 Rico *et al.*, 2007
Luz pulsada	- Reduções microbianas significativas com tratamentos de curto período de tempo - Eficaz em alimentos sólidos e líquidos - Ausência de resíduos - Alguns componentes do alimento podem absorver a radiação impedindo a sua absorção pelos microrganismos - Eficácia reduzida a níveis de contaminação altos; Possível resistência de alguns microrganismos - Ocorrência de efeitos químicos adversos nos alimentos	[6-12 J/cm^2] Redução: 1,56-3,01 log ufc/g	Oms-Oliu *et al.*, 2010 Ramos *et al.*, 2013 Ramos-Villarroel *et al.*, 2012
Alta pressão (High Pressure Proceding – HPP)	- Inativação microbiana e enzimática - Não provoca degradação do sabor e dos nutrientes; Não há evidência de toxicidade - Equipamento dispendioso - Os alimentos devem conter cerca de 40% de água livre para haver efeito antimicrobiano - Não é eficiente nas enzimas da fruta	[50-1000 MPa]	Ramos *et al.*, 2013
Ultra-sons	- Aumento da taxa de transferência de calor - Redução do tempo e da temperatura de processamento - Deve ser aplicado em conjunto com outros tratamentos - Dificuldade no *scale-up* do processo - Alterações na estrutura e textura do alimento - Penetração no alimento é afetada pela presença de sólidos e de ar	[20-100 kHz]	Chemat *et al.*, 2011 Kiani *et al.*, 2014 O'Donnel *et al.*, 2010 Ramos *et al.*, 2013

Um dos objetivos deste trabalho foi estudar a aplicação de técnicas alternativas ao cloro na desinfeção de FMP, utilizando como método químico a água electrolisada (ácida e neutra) e o como método físico a iluminação UV-C. Assim, apresenta-se seguidamente a descrição destes métodos e alguns exemplos da sua aplicação na desinfeção de fruta.

Água electrolisada

Existem dois tipos de água electrolisada (AE) que apresentam propriedades desinfetantes, a água electrolisada ácida (AEA) e a água electrolisada neutra (AEN). A AE é formada através da eletrólise de uma solução saturada de cloreto de sódio (NaCl) numa célula eletrolítica. A solução passa por dois canais, o ânodo (+) e o cátodo (-), onde é exposta a um diferencial elétrico controlado. Durante esta passagem ocorre uma acumulação de iões de cloro no ânodo, formando uma solução ácida (AEA) e uma acumulação de iões de sódio e hidróxido no cátodo, formando uma solução alcalina (AEN). A AEA tem-se mostrado eficiente na eliminação de microrganismos patogénicos de origem alimentar *in vitro* e em reduzir as contagens de microrganismos em frutas e vegetais. Este efeito é atribuído ao seu baixo pH (2-4) ao seu elevado potencial de oxidação-redução (acima de 1000 mV) e ao seu conteúdo em substâncias oxidantes como é o caso do ácido hipocloroso (HOCl) (Kim *et al*., 2000). A AEN também possui um forte efeito bactericida, com valores de pH de 5,0 a 8,5 e com um potencial de oxidação-redução de 500 a 700 mV. Os principais reagentes biocidas da AEN são HOCl, OCl^-, o radical hidroxiperoxilo (HO^{-2}) e o radical superóxido ($^{\bullet}O_2$) (Abadias *et al*., 2008a). O modo de ação da AE ainda não está esclarecido, contudo pensa-se que a sua atividade antimicrobiana está relacionada com alterações a nível da parede celular dos microrganismos (Osafune *et al*., 2006) e com o potencial oxidante do HOCl através da produção de radicais hidroxilo ($^{\bullet}OH$) os quais atuam nos componentes celulares tais como proteínas e ácidos nucleicos (Huang *et al*., 2008).

Vários estudos têm demonstrado a eficácia da AE na eliminação de microrganismos de frutos e vegetais. A lavagem de maçãs minimamente processadas com AEA ou AEN com 100 e 50 ppm de cloro livre, permitiu obter reduções nas populações de *Escherichia coli*, *Salmonella* Choleraesuis e de *Listeria innocua* semelhantes ou superiores à lavagem com soluções de hipoclorito de sódio com as mesmas concentrações (Graça *et al*., 2011, 2012). Graça *et al*. (2010a,b) observou que a lavagem, durante 5 min, com AEA e AEN com 200 ppm de cloro livre reduziu a população de *L. innocua* mais de 1 ciclo logarítmico em amostras de maçã minimamente processada e cerca de 1 log ufc/g em laranjas minimamente processadas. No mesmo estudo, foi observada uma redução da população de *E. coli* de 2,05 log ufc/g e de 1,70 log ufc/g após a lavagem de amostras de laranja cortada com AEA e AEN com a mesma concentração, respetivamente. Abadias *et al*. (2008a) observaram reduções nas contagens de *E. coli* O157:H7, *Salmonella*, *L. innocua* e *Erwinia carotovora* de cerca de 1-2 log ufc/g em alface após a lavagem com AEN com uma concentração de 50 ppm de cloro livre. Izumi (1999) observou reduções nas contagens microbianas entre 0,6 e 2,6 log ufc/g em vegetais cortados após a lavagem durante 3 min com AEN com 50 ppm de cloro livre. Ding *et al.* (2015) realizaram um estudo em que desinfetaram a superfície de tomate cherry e de morangos com AE a pH 6,49. As reduções nas populações de microrganismos obtidas nesse estudo foram de 1,45 log ufc/g e de 1,10 log ufc/g nas populações de bactérias aeróbias totais e bolores e leveduras, respetivamente, em tomate cherry e de 0,93 e 0,96 log ufc/g nas populações de bactérias aeróbias totais e bolores e leveduras, respetivamente, em morangos.

Embora o cloro seja o principal responsável pelo seu efeito microbicida, a AE apresenta algumas vantagens em relação ao uso de soluções comerciais de cloro, como o hipoclorito de sódio. A AE tem como vantagens possuir uma eficácia antimicrobiana superior ao cloro por apresentar um potencial de oxidação redução mais elevado (Jemni *et al.*, 2014; Rico *et al.*, 2007) e pode ser produzida no local e no momento em que irá ser aplicada, fazendo com que não seja necessário o armazenamento de compostos potencialmente perigosos como o hipoclorito de sódio (Jemni *et al.*, 2014).

As vantagens da AEN residem no facto do valor do seu pH ser de 5,0 a 8,5 não sendo tão corrosiva para os equipamentos, nem irritante para a pele, sendo também mais estável relativamente à perda de cloro (Abadias *et al.*, 2008a; Jemni *et al.*, 2014). As desvantagens da AEA incluem a formação de cloro gasoso durante a sua produção, o que pode ser nocivo para os operadores e o seu baixo valor de pH torna-a corrosiva para os equipamentos (Huang *et al.*, 2008).

Iluminação Ultravioleta-C

A luz Ultravioleta (UV) está compreendida numa banda de comprimentos de onda da região não-ionizante do espectro eletromagnético, entre os raios-X (200 nm) e a luz visível (400 nm). O espectro UV pode ser dividido em três regiões: ondas curtas de UV (UV-C) com comprimentos de onda de 200 a 280 nm; ondas médias de UV (UV-B) com comprimentos de onda de 280 a 320 nm; e ondas longas de UV (UV-A) com comprimentos de onda entre 320 e 400 nm. A intensidade da iluminação UV é expressa como fluxo de intensidade (W/m^2) e a dose, que é em função da intensidade e tempo de exposição, é expressa como radiação de exposição (J/m^2) (Bintsis *et al.*, 2000). A iluminação UV-C tem o máximo efeito germicida no comprimento de onda de 254 nm e é letal para a maioria dos microrganismos (bactérias, protozoários, fungos e algas) e vírus (Bintsis *et al*., 2000).

O modo de ação da iluminação UV-C (0,5-20 kJ/m^2) na inibição do crescimento microbiano deve-se à indução da formação de dímeros de pirimidinas que provocam a distorção da hélice de ADN bloqueando a replicação das células microbianas, causando a sua morte (Jemni *et al*., 2014; Kim *et al*., 2013). No entanto, existem sistemas de reparação, como sistemas enzimáticos de foto reparação, genes de reparação-excisão induzidos por raios UV que restauram a integridade do ADN das células microbianas expostas à iluminação UV (Cleaver, 2003; Pruteanu e Baker, 2009).

O efeito da iluminação UV-C é independente da temperatura a que é aplicada (num intervalo de 5 a 37 °C) mas depende da incidência da irradiação que é determinada pela estrutura e topografia do produto irradiado (Bintsis *et al*., 2000), do fluxo de radiação e da posição entre a fonte de iluminação e a amostra irradiada (Escalona *et al*., 2010). A iluminação UV-C pode também exercer um efeito indireto sobre os microrganismos ao estimular mecanismos de defesa no produto tratado, provocando um atraso na senescência e degradação do produto. Estes efeitos podem, se ultrapassarem certos limites, resultar em defeitos tal como se refere na página seguinte a propósito das desvantagens da iluminação UV-C. (Escalona *et al*., 2010; Jemni *et al*., 2014). O ponto crítico deste processo consiste em encontrar uma dose que permita afetar o crescimento dos microrganismos patogénicos sem danificar o alimento (Ben-Yehoshua e Mercier, 2005; Gómez-López *et al*., 2007; Manzocco *et al*., 2011).

A iluminação UV-C tem sido utilizada com sucesso para reduzir a contaminação microbiana e/ou prolongar o tempo de prateleira em frutos inteiros (Nunes, 2010) e frutos cortados como manga e ananás (George *et al*., 2015), melancia (Artés-Hernández *et al*., 2010), kiwi (Beirão da Costa *et al*., 2014), maçãs (Graça *et al*., 2013), alperces (Yun *et al*., 2013), laranja (Salazar *et al*., 2010) e melão (Manzocco *et al*., 2011). Manzocco *et al*. (2011) observaram reduções acima de 2 log ufc/g nas contagens de microrganismos viáveis totais e de enterobactérias em melão minimamente processado após o tratamento com iluminação UV-C com as doses de 1200, 6000 e 12000 J/m^2. Artés-Hernández *et al*. (2010) constataram que melancia minimamente processada submetida a tratamentos de iluminação UV-C (1,6, 2,8, 4,8 e 7,2 kJ/m^2) após 11 dias de armazenamento a 5 °C apresentava populações de microrganismos mesófilos, psicrotróficos e de enterobactérias menores do que as amostras controlo. Fonseca e Rushing (2006) submeteram embalagens de melancia minimamente processada a iluminação UV-C (4,1 kJ/m^2) e observaram uma redução das populações microbianas acima de 1 log, após 7 dias de armazenamento. A iluminação UV-C tem sido também associada ao aumento da atividade antioxidante em mangas e em ananás (George *et al*., 2015) e em melancia (Artés-Hernández *et al*., 2010), romã (Nunes *et al*., 2010), ao aumento da atividade da peroxidase em melão Cantaloupe (Lamikanra *et al*., 2005), na indução da produção de antocianinas e de estilbenos (Ramos *et al*., 2013) e no aumento da estabilidade enzimática de frutas minimamente processadas através da inativação da enzima pectinolítica pectato liase (Manzocco *et al*., 2009a) e de polifenoloxidases (Manzocco *et al*., 2009b) em maçãs.

Entre as vantagens desta técnica estão o seu fácil manuseamento, os custos reduzidos do equipamento, os gastos de energia e manutenção e a ausência de resíduos nos alimentos (Kim *et al*., 2013; Manzocco *et al*., 2011). A principal desvantagem da luz UV-C é não ter poder de penetração nos alimentos líquidos ou sólidos, sendo por esta razão apenas eficiente enquanto método de desinfeção superficial (Manzocco *et al*., 2011). Outras desvantagens do uso da iluminação UV-C são a possível indução de stresse na fruta e o aumento da sua respiração o que pode provocar alterações na cor e produção de *off-flavors* (Manzocco *et al*., 2011; Ramos *et al*., 2013).

1.3. Qualidade microbiológica de fruta minimamente processada

Um dos fatores responsáveis pelo aumento de doenças do foro alimentar associadas ao consumo de vegetais e frutos foi o aumento do consumo de produtos minimamente processados (Tabela 1.3). A segurança microbiológica e o prolongamento do período de vida útil destes alimentos são dois dos principais desafios que a indústria enfrenta (Ölmez e Kretzschmar, 2009; Parish *et al*., 2003; Ramos *et al*., 2013). A fruta fresca minimamente processada caracteriza-se por possuir superfícies cortadas, não esterilizadas, fisiologicamente ativas, ricas em nutrientes e água. Por esta razão, são alimentos suscetíveis à contaminação microbiológica, nas diversas fases, desde o seu processamento até à sua distribuição não descurando o manuseamento em casa dos consumidores. São alimentos crus, prontos a consumir, não sendo submetidos a qualquer processo de preservação térmico ou químico (adição de conservantes). Esta fruta pode conter diversos microrganismos que estão presentes naturalmente sobre a fruta, ou são adquiridos durante as operações de pré-colheita, colheita, processamento mínimo e manuseamento em condições de higiene deficiente (Abadias *et al*., 2008b; Graça *et al*., 2015; Olaimat e Holley, 2012). Se as operações de processamento forem impróprias e o armazenamento e distribuição ocorrerem em condições inadequadas (temperatura, humidade relativa), alguns microrganismos constituintes da população inicial, poderão sobreviver e multiplicar-se. Caso se trate de microrganismos de degradação aceleram os processos de degradação, se microrganismos patogénicos aumentam o risco do alimento se tornar um perigo para a saúde pública. A tendência para aumentar o tempo de vida útil dos alimentos refrigerados pode permitir o crescimento de microrganismos patogénicos psicrotróficos tais como *L. monocytogenes* (Melo *et al*., 2015).

Tabela 1.3. Fatores associados ao aumento de número de infeções causadas pela ingestão de alimentos de origem vegetal (Beuchat, 2002, Brandl, 2006, Tauxe *et al*., 1997).

Alterações na indústria: Intensificação e centralização da produção Redes de distribuição muito grandes Práticas de higiene insuficientes Introdução na cadeia alimentar de alimentos minimamente processados (frutos e vegetais) Aumento das importações de frutos e vegetais
Alterações nos hábitos dos consumidores/ Alterações no estilo de vida Aumento do consumo de refeições fora de casa Aumento do consumo de frutos e vegetais frescos Aumento do consumo de sumos de fruta frescos
Aumento da dimensão das populações de risco (idosos, imunodeprimidos)
Vigilância epidemiológica intensificada
Desenvolvimento de novos métodos para identificar e diagnosticar os microrganismos patogénicos
Aparecimento de microrganismos patogénicos emergentes com doses infeciosas baixas
Evolução dos microrganismos e alterações nas características de patogenicidade e virulência

Por outro lado, a utilização de técnicas alternativas de embalamento como a atmosfera modificada contribui para inibir o crescimento dos microrganismos aeróbios, degradadores da fruta, mas não inibe microrganismos patogénicos anaeróbios e anaeróbios facultativos que têm a capacidade de sobreviver e multiplicar-se nestas condições (Hurst, 2002).

Desta forma, a fruta minimamente processada constitui um meio ótimo para a sobrevivência e o crescimento de microrganismos patogénicos pois as suas barreiras naturais de proteção foram removidas durante o seu processamento pelo que o seu consumo tem sido associado a infeções provocadas por bactérias, vírus e parasitas (Heaton e Jones, 2008; Olaimat e Holley, 2012; Ramos *et al*., 2013; Strawn *et al*., 2011). Os principais microrganismos patogénicos associados ao consumo de alimentos de origem vegetal incluem *S. enterica, E. coli, L. monocytogenes, Campylobacter jejuni, Shigella* spp., *Clostridium botulinum* e *C. perfringens, Vibrio*. Os vírus da Hepatite A e Norovirus são cada vez mais associados a doenças transmitidas por FCF (Strawn *et al*., 2011; Harris *et al*., 2003; Oliveira *et al*., 2015). Contudo, *S. enterica*, *E. coli*, *L. monocytogenes* e *C. jejuni* são os principais agentes etiológicos, mais comumente, identificados durante os surtos associados ao consumo de frutos (Tabela 1.4).

1.3.1. *Salmonella enterica*

O género *Salmonella*, incluído na família *Enterobacteriaceae*, contém duas espécies, *S. bongori* e *S. enterica* que se encontra subdividida em seis subespécies: *enterica*, *salamae*, *arizonae*, *diarizonae*, *houtenae* e *indica*. As bactérias deste género são diferenciadas com base nas variações em antigénios, os antigénios somáticos presentes nos lipopolissacáridos da parede celular (antigénio O), os antigénios presentes na proteína flagelar (flagelina) (antigénios H1 e H2), e os antigénios capsulares (Vi) dando origem a mais de 2500 combinações dos antigénios e consequentemente a um número muito elevado de serotipos/serovares (Grimont e Well, 2007; McQuiston *et al*., 2004). Relativamente a *S. enterica* subespécie *enterica* estão descritos mais de 1500 serotipos.

São bactérias com a forma de bastonetes Gram negativas, anaeróbios facultativos, na sua maioria móveis por flagelos perítricos, possuem catalases mas dão origem a um teste da oxidase negativo. Tratam-se de bactérias intracelulares facultativas encontradas numa série de células fagocíticas e não fagocíticas.

Dependendo do hospedeiro e do serotipo, *S. enterica* é o agente etiológico de febre entérica (febre tifoide), enterocolite/diarreia e bacteriemias. Existem também os portadores crónicos de *Salmonella* (Coburn *et al*., 2007). Os serotipos *S.* Typhi, *S.* Paratyphi estão particularmente adaptados aos humanos, causando febre tifoide. Os serotipos de *Salmonella* não tifoide são os agentes mais frequentes de salmoneloses nos países desenvolvidos. O serotipo *S.* Choleraeseuis, cujo hospedeiro primário é o porco, causa também doença severa em humanos e os serotipos ubíquos *S.* Enteritidis e

S. Typhimurium causam infeções gastrointestinais em humanos podendo induzir doenças noutros animais (Hoelzer *et al.*, 2011).

Tabela 1.4. Surtos associados ao consumo de fruta.

Agente	**Fruta**	**Ano**	**Local**	**Referência**
Salmonella Weltevreden	Ananás, melancia, papaia MP	1996	Singapura	Ooi, 1997
Salmonella Newport	Manga	1999	EUA	Sivapalasingam *et al.*, 2004
Salmonella Saintpaul	Manga	2001	EUA	Beatty *et al.*, 2004
Salmonella Litchfield	Papaia MP	2006	Austrália	Gibbs *et al.*, 2009
Salmonella Litchfield	Melão Cantaloupe	2008	EUA	CDC, 2008b
Salmonella Agona	Papaia	2011	EUA	CDC, 2011a
Salmonella Panama	Melão Cantaloupe	2011	EUA	CDC, 2011b
Salmonella Braenderup	Manga	2012	EUA e Canadá	CDC, 2012a PHAC, 2012
Salmonella Typhimurium e *Salmonella* Newport	Melão Cantaloupe	2012	EUA	CDC, 2012b
Salmonella Newport	Melancia	2012	UE	Byrne *et al.*, 2014
Escherichia coli O157:H7	Melão Cantaloupe	1993	EUA	Sivapalasingam *et al.*, 2004
E. coli O11:H43	Ananás	1994	EUA	Sivapalasingam *et al.*, 2004
E. coli O157:H7	Uvas	2000	EUA	CSPI
E. coli O157:H7	Pera	2001	EUA	CSPI
E. coli O26	Morangos e mirtilos	2006	EUA	CSPI
E. coli O157:H7	Melão Cantaloupe	2008	EUA	CSPI
E. coli O157:H7	Morango	2011	EUA	FDA, 2011
Campylobacter jejuni	Salada de frutas	1994	EUA	CSPI
C. jejuni	Salada de frutas	2001	EUA	CSPI
C. jejuni	Melão e melancia	2006	EUA	CSPI
L. monocytogenes	Melão Cantaloupe	2011	EUA	CDC, 2011c
Hepatite A	Sementes de romã	2013	EUA	CDC, 2013
Hepatite A	Morango e manga	2013	UE	ECDC, 2014
Norovirus	Ananás MP	2001	EUA	Sivapalasingam *et al.*, 2004
Norovirus	Abacate	2001	EUA	CSPI
Norovirus	Ananás, banana e melão Cantaloupe	2003	EUA	CSPI
Norovirus	Ananás e melão Cantaloupe	2011	EUA	CSPI

Os serotipos *S.* Choleraeseuis e *S.* Dublin estão mais comumente associadas a bacteriemia (Fierer e Guiney, 2001). Existem serotipos exclusivamente adaptados a animais como *S.* Gallinarum que é específica de aves, sendo responsável por infeções sistémicas nestes animais (Hoelzer *et al*., 2011). Alguns serotipos têm sido associados a clorose de folhas de plantas causando a morte das folhas pois têm capacidade de aderir à sua superfície e a alguns dos seus órgãos internos (Gu *et al*., 2013; Klerks *et al*., 2007; Schikora *et al*., 2008). Diversos estudos têm evidenciado a capacidade de *Salmonella* spp. de se internalizar em tecidos vegetais através das raízes, estomas, lenticelas e lesões existentes nos órgãos da planta (Brandl, 2006) e a de se multiplicar na superfície de folhas de plantas jovens (Brandl e Amundson, 2008). Este tipo de trabalhos têm alertado para a possibilidade das plantas poderem constituir hospedeiros alternativos para *Salmonella* e terem um papel importante na sua transmissão aos animais (Melotto *et al*., 2014; Wiedemann *et al*., 2015).

O reservatório comum de *S. enterica* é o trato gastrointestinal do Homem, animais domésticos e selvagens. *S. enterica* entra nos solos e ambientes agrícolas através das fezes dos animais, podendo contaminar diretamente as plantas e as águas superficiais utilizadas na irrigação e na preparação de pesticidas e fertilizantes. A forma de transmissão principal de *S. enterica* é a via oral-fecal, podendo também ocorrer outras formas de contaminação. Os manipuladores infetados, incluindo os que não manifestam sintomas (portadores assintomáticos), podem ser uma fonte de contaminação dos alimentos assim como a contaminação cruzada de alimentos prontos a consumir.

De acordo com o serotipo e os seus fatores de virulência, a quantidade de inóculo e o estado imunológico do hospedeiro, *S. enterica* pode provocar uma vasta gama de doenças que incluem gastroenterites ligeiras até infeções sistémicas graves como bacteriemias, osteomielites e meningites. Alguns pacientes podem desenvolver artrites crónicas. As formas de salmoneloses mais graves afetam fundamentalmente grupos de risco, tais como os idosos, os indivíduos imunocomprometidos e as crianças (Batz *et al*., 2013; Hurley *et al*., 2014).

As características de virulência de *Salmonella* residem na capacidade das bactérias invadirem as células eucariotas, se evadirem da fagocitose e sobreviverem no interior de células fagocíticas e não fagocíticas do hospedeiro onde têm capacidade de se multiplicar tornando-se um microrganismo intracelular (Coburn *et al*., 2007). As salmoneloses ocorrem após a ingestão de alimentos ou água contaminados com estirpes patogénicas ou em resultado do contacto com um paciente infetado ou um portador assintomático. As bactérias sobrevivem à acidez do estômago e colonizam o intestino onde invadem as células epiteliais fagocíticas. A internalização de células não fagocíticas pode ocorrer através de dois mecanismos de invasão que envolvem a modulação do citoesqueleto de actina, o “Zipper” e o “Triger” (Boumart *et al*., 2014). Em qualquer dos casos, a internalização das bactérias ocorre através da formação de um vacúolo que contém os microrganismos e onde têm capacidade de se multiplicar. Contudo, os vacúolos contendo as bactérias podem ser destruídos por ligação a um lisossoma ou em resultado de uma resposta autofágica, libertando as bactérias para o citosol das células hospedeiras. No citosol de células epiteliais pode ocorrer replicação evidenciando que *Salmonella* pode manifestar um estilo de vida intracelular (Malik-Kale *et al*., 2012). Os genes que contêm informação genética para os complexos mecanismos através dos quais as bactérias atuam como microrganismos patogénicos de extrema virulência estão localizados nas ilhas de patogenicidade de *Salmonella*.

As salmoneloses têm uma incidência muito elevada na Europa (EFSA, 2014) e nos EUA (CDC, 2013b), constituindo um problema económico e de saúde pública. No entanto, na Europa em 2012 foram reportados 92.916 casos de salmoneloses, correspondendo a um declínio de cerca de 4,7 % em comparação com o número de ocorrências registadas no ano anterior e de 32 % relativamente a 2008. As serovares mais frequentemente associadas aos referidos casos de salmoneloses foram *S.* Enteritidis e *S.* Typhimurium. No que diz respeito aos surtos, observou-se também uma redução de cerca de 19 % de 2008 a 2012. Os principais alimentos envolvidos em surtos foram os ovos e outros alimentos contendo ovos, queijo e carne de aves (EFSA, 2014). *S. enterica* é o agente etiológico mais comummente associado a infeções e surtos provocadas pela ingestão de alimentos de origem vegetal frescos (Heaton e Jones, 2008) sumarizados na Tabela 1.4.

De acordo com os critérios microbiológicos da União Europeia (Regulamentos CE 2073/2005 e 1441/2007) *S. enterica* deve estar ausente nas diversas categorias de alimentos incluindo nos frutos e vegetais minimamente processados. Dos alimentos estudados, aqueles em que foram encontradas as mais altas proporções de não conformidades com os critérios microbiológicos foram as carnes de frango, de peru, de porco, de bovino e os ovos. Contudo, algumas amostras de vegetais e frutas minimamente processados, reportadas em alguns estados membros, revelaram não respeitar os critérios microbiológicos (EFSA, 2014).

Se houver contaminação de origem fecal dos alimentos, a transmissão dos microrganismos ocorrerá, particularmente em condições que favoreçam o seu crescimento, no caso dos alimentos serem acondicionados a temperaturas inadequadas de conservação, se não tiverem sido cozinhadas durante o tempo adequado. Strawn e Danyluk (2010) observaram um crescimento na população de *Salmonella* spp. em mangas e papaias cortadas, armazenadas a 12 °C e a sua sobrevivência durante 28 dias quando as amostras foram armazenadas a 4 °C. Observaram ainda a sobrevivência de *Salmonella* spp. nas mangas e papaias cortadas após 180 dias de congelamento. Um aumento nas populações de *Salmonella* em pêssegos e maçãs minimamente processados armazenados a 20 e a 25 °C foi também observado por Alegre *et al*. (2010a; 2010b). Ma *et al*. (2016) observaram que a população de *Salmonella* inoculada em maçãs, goiaba, banana, pitaia, manga e ananás minimamente processados teve a capacidade de sobreviver durante 6 dias a 4 °C e Ukuku *et al*. (2015) observaram a sobrevivência da população de *Salmonella* em melão Cantaloupe cortado armazenado a 5 °C durante 7 dias e um aumento da sua taxa específica de crescimento nas amostras armazenadas a 10, 15 e 20 °C. Sim *et al*. (2013) reportaram um aumento superior a 2,0 log ufc/g da população de *Salmonella* spp. inoculada em pitaias minimamente processadas após um armazenamento a 28 °C durante 2 dias. Nas amostras a 12 °C observaram um aumento de cerca de 0,9 log ufc/g e nas amostras armazenadas a 4 °C não houve crescimento mas constataram que a população de *Salmonella* spp. teve a capacidade de sobreviver durante os 4 dias de armazenamento. Palekar *et al*. (2015) observaram a sobrevivência de *Salmonella* Poona durante 21 dias em fatias de melão Cantaloupe armazenadas a 5 °C.

1.3.2. *Escherichia coli*

Escherichia coli é uma espécie da família *Enterobacteriaceae*, em que as células apresentam a forma de bastonete Gram negativo, possuem catalases e dão origem a um teste da oxidase negativo. Estas células podem crescer aeróbia ou anaerobiamente, de preferência a 37 °C, podendo ser móveis com flagelos perítricos ou não móveis. *E. coli* integra a microbiota comensal do trato gastrointestinal do Homem e numerosos mamíferos. Contudo, têm sido descritas diversas estirpes de *E. coli* responsáveis por infeções entéricas e extraintestinais (Ex.: infeções do trato urinário, sepsis e meningite neonatal), associadas a morbilidade e mortalidade elevadas em todo o mundo (Clements *et al.*, 2012). Relativamente às estirpes entéricas estão descritos 7 patotipos de *E. coli* de acordo com a natureza da doença que causam, com os fatores de virulência que possuem e com o seu perfil filogenético: *E. coli* enteropatogénica (EPEC) que foi a primeira estirpe associada a surtos diarreicos em 1940 e 1950; *E. coli* produtora de toxina Shiga (verocitotoxinas) (STEC) [Ex. *E. coli* enterohemorrágica (EHEC) *E. coli* O157:H7]; *E. coli* enteroinvasiva (EIEC) que possui mecanismos de virulência e infeções semelhantes a *Shigella*; *E. coli* enteroagregativa (EAEC) (Ex.: *E. coli* O104:H4, responsável pelo surto que ocorreu na Alemanha em 2011); *E. coli* difusamente aderente (DAEC); *E. coli* enterotoxicogenica (ETEC) que é a causa mais comum de diarreia do viajante e *E. coli* aderente invasiva (AIEC) que tem sido associada à doença de Crohn (Borgatta *et al*, 2012; Clements *et al.*, 2012; Croxen *et al.*, 2013; van Elsas *et al.*, 2011). A espécie *E. coli* encontra-se em permanente evolução o que é demonstrado pela existência de estirpes “híbridas” tais como *E. coli* O104:H4 que, apesar de ter sido incluída no grupo EAEC, possui características de estirpes enterohemorrágicas típicas do grupo STEC, tendo alguns autores proposto a sua inclusão num novo grupo (STEAEC) (Croxen *et al.*, 2013; van Elsas *et al.*, 2011). Algumas particularidades dos patotipos de *E. coli* estão resumidas na Tabela 1.5.

Tabela 1.5. Algumas características dos 7 patotipos de *E. coli* (Croxen *et al.*, 2013).

Patotipos	**Hospedeiro**	**Local da colonização**	**Doença**	**Reservatório conhecido** **Fonte de contaminação**
E. coli **EPEC**	Crianças <5 anos, adultos (dose infeciosa alta)	Intestino delgado	Diarreia abundante	Humanos e animais
E. coli **STEC**	Adultos e crianças (dose infeciosa baixa)	Ílio distal, cólon	Diarreia aquosa Colite hemorrágica HUS - Síndrome hemolítico urémico	Humanos, animais, alimentos e água
E. coli **EIEC**	Crianças <5 anos, adultos Viajantes Pacientes imunocomprometidos (dose infeciosa baixa-10-100 ufc)	Cólon	Disenteria HUS (potencial)	Humanos, animais, alimentos e água
E. coli **EAEC**	Adultos Crianças	Intestino delgado e/ou cólon	Diarreia do viajante HUS Diarreia persistente	Alimentos Portadores humanos
E. coli **ETEC**	Pacientes imunocomprometidos Crianças <5 anos Viajantes (dose infeciosa alta- 10^6-10^8 microrganismos)	Intestino delgado	Diarreia do viajante Diarreia persistente Diarreia aquosa	Alimentos, água, humanos e animais
E. coli **DAEC**	Crianças Adultos	Intestino (localização não caracterizada)	Diarreia persistente aquosa (crianças) Associada a Doença de Crohn em adultos	Desconhecido
E. coli **AIEC**	Adultos Crianças	Intestino delgado	Associada a doença de Crohn	Desconhecido

A patogenicidade e virulência de *E. coli* têm sido explicadas pela aquisição de uma série de fatores de virulência relacionados com a aderência/colonização, a invasão, o controlo dos processos fisiológicos das células hospedeiras, a secreção de toxinas e a produção de sideróforos (Touchon *et al.*, 2009). Estes fatores de patogenicidade e virulência encontram-se em regiões do genoma (ilhas de patogenicidade genómica). Os tamanhos dos genomas de *E. coli* comensal e dos diferentes patotipos podem diferir entre si em cerca um milhão de pares de bases. Existe um conjunto de genes conservado comum designado "genoma core" e um conjunto flexível de genes. A patogenicidade é conferida pelo conjunto flexível de genes adquirido do exterior podendo também haver perda de genes. A aquisição ou perda de genes pode ocorrer por conjugação, transformação e transdução ou resultante do movimento de elementos genéticos móveis (transposões, sequências de inserção, bacteriófagos e plasmídeos) por transferência horizontal de genes [Horizontal gene transfer (HGT)]. Por outro lado, a maior parte dos genes das toxinas e dos fatores de colonização (CF) requeridos para a patogenicidade de algumas estirpes (por exemplo, ETEC) encontram-se quase exclusivamente em plasmídeos (Clements *et al.*, 2012; Croxen *et al.*, 2013; van Elsas *et al.*, 2011). Na Tabela 1.6 estão resumidas algumas características de virulência e patogenicidade, nomeadamente, de adesão às células do hospedeiro e produção de toxinas, das estirpes patogénicas de *E. coli*.

As estirpes de *E. coli* têm como reservatório o trato gastrointestinal do Homem e de outros animais. A matéria fecal pode contaminar os alimentos e a água, incluindo águas de irrigação e águas recreativas. As infeções humanas por estirpes de *E. coli* patogénicas ocorrem após o consumo de alimentos contaminados tais como carne mal cozinhada, alimentos frescos de origem vegetal contaminados ou através do contacto com animais contaminados. Pode ocorrer também transmissão da doença através do contacto pessoa-pessoa, quando os devidos cuidados de higiene não são assegurados. Os alimentos de origem vegetal, incluindo os frutos, podem ser contaminados por contaminação cruzada através do contacto com carne crua contaminada. Indivíduos manipuladores de alimentos (portadores sintomáticos ou assintomáticos) podem também ser responsáveis pela transmissão das bactérias (Berger *et al* 2010; Croxen *et al.*, 2013). De acordo com o relatório da EFSA (2014), na Europa os principais alimentos transmissores destas bactérias foram as carnes de bovino e ovino. No entanto os alimentos de origem vegetal e as águas revelaram-se importantes fontes de contaminação.

Tabela 1.6. Alguns mecanismos de virulência (adesão e toxinas) das estirpes de *E. coli* patogénicas (Clements *et al.*, 2012; Croxen *et al.*, 2013).

Patotipos	**Adesão**	**Toxinas**	**Observações**
E. coli **EPEC**	Intiminas Pili Adesinas Fímbrias polares longas (LPF)	-	Tem capacidade de formar micro-colónias sobre as microvilosidades Formação do pedestal de actina nas células epiteliais do lúmen
E. coli **STEC**	Intiminas, Adesinas, Toxina B Fator de aderência de *E. coli* Fímbrias polares longas (LPF) Adesina autoaglutinativa (Saa) Adesina homóloga (LHA)	Toxina Shiga (Stx)	Tem capacidade de formar células viáveis não cultiváveis
E. coli **EIEC**	-	Enterotoxina 1 e 2 (de *Shigella*) (ShET1/2)	Microrganismo patogénico intracelular facultativo
E. coli **EAEC**	Fimbrias de aderência agregativa (AAF) Locus de invasão toxicogénica (Tia)	Enterotoxina estável ao calor (EAEC) Hemolisina E Enterotoxina 1 e 2 (de *Shigella*) (ShET1/2) Toxina Shiga (Stx)	Forma biofilmes sobre a mucosa intestinal
E. coli **ETEC**	Fatores de colonização (CF) Adesinas	Enterotoxina sensível ao calor (LT) Enterotoxina estável ao calor (ST) Citolisina A (CIyA)	
E. coli **DAEC**	Fímbrias Adesinas	-	Adere difusamente sobre as células intestinais
E. coli **AIEC**	Pili Fímbrias polares longas (LPF)	-	Invade as células epiteliais de pacientes com doença de Crohn. Replica-se no interior de células epiteliais e macrófagos

O referido relatório indica que as infeções causadas por estipes de *E. coli* produtoras de verocitotoxinas têm vindo a aumentar na Europa, de 2008 a 2012, tendo-se observado um pico em 2011, resultante do surto ocorrido na Alemanha após o consumo de sementes germinadas. Em 2012, foram confirmados cerca de 5670 casos de infeção por VTEC sendo, a maior parte, casos esporádicos. Nos EUA, as estirpes produtoras de verocitotoxinas, nomeadamente *E. coli* O157:H7, tem sido responsabilizada por um elevado número de infeções e os surtos reportados recentemente foram atribuídos ao consumo de vegetais, frutas/frutos secos, produtos lácteos e carne moída (CDC, 2013b).

A capacidade das estirpes de *E. coli* sobreviverem e cresceram em ambientes diferentes dos do trato gastrointestinal resulta num problema grave de saúde pública. Observou-se que *E. coli* foi isolada de ambientes como o solo, o estrume e água de irrigação e revelou capacidade de colonizar compartimentos internos de alface (Solomon *et al*., 2002) e raízes de plantas (Natvig *et al*., 2002). Assim, estes produtos podem contribuir para a disseminação das bactérias em ambientes industriais de processamento e embalamento de alimentos contribuindo para a sua disseminação na cadeia alimentar, se não forem respeitados as boas práticas de fabrico. *E. coli* O157:H7 tem a capacidade de sobreviver e de se multiplicar fora do intestino e foi implicada em vários surtos de doença relacionados com a ingestão de frutas e vegetais minimamente processados (Oliveira *et al*., 2015). Abadias *et al*. (2012) constataram a sobrevivência da população de *E. coli* O157:H7 inoculada em ananás e melão minimamente processados, embalados em atmosfera modificada e armazenados a 5 °C durante 15 dias. Em pêssegos e maçãs minimamente processados foi observado um aumento, acima de 2 log ufc/g, na população de *E. coli* O157:H7, às temperaturas de 20 e 25 °C (Alegre *et al*., 2010a e 2010b). Strawn e Danyluk (2010) observaram que *E. coli* O157:H7 teve a capacidade de crescer em amostras de manga e papaia minimamente processadas armazenadas a 23 °C, de sobreviver durante 28 dias quando as amostras foram armazenadas a 4 °C e 180 dias quando as amostras foram congelas a -20 °C.

1.3.3. *Listeria monocytogenes*

Listeria monocytogenes está incluída no género *Listeria* conjuntamente com mais 7 espécies, *L. innocua*, *L. seeligeri*, *L. welshimeri*, *L. grayi*, *L. ivanovii*, *L. rocourtiae* e *L. marthii* (Graves *et al*., 2010; Leclercq *et al*., 2010; Liu, 2006). *L. monocytogenes* está associada a doença em humanos e noutros animais enquanto *L. ivanovii* tem sido, fundamentalmente, implicada em doenças nos ruminantes (Hain *et al*., 2006).

As células de *L. monocytogenes* apresentam-se com uma forma de bastonetes curtos com dimensões entre 0,5 a 2 µm, Gram positivas, possuidoras de catalase e teste de oxidase negativo, não formadoras de esporos e microaerofílicas. Estas bactérias são imóveis a 37 °C mas possuem uma mobilidade característica a 20-25 °C que consiste em movimentos de rotação sobre o seu próprio eixo. É uma espécie com uma temperatura ótima de crescimento de 30-37 °C mas com capacidade de tolerar temperaturas de 45 °C. Devido ao facto de crescer a temperaturas de refrigeração (4 °C) é classificada como microrganismo psicrotrófico (Melo *et al*., 2015).

É um microrganismo saprófita, presente no solo, na água, nos efluentes e em material vegetal em decomposição (Thevenot *et al*., 2006). Tem também sido isolado de fezes de humanos (Grif *et al*., 2003; Ooi e Lorber, 2005). A sua presença tem sido identificada em diversos alimentos crus de origem vegetal (frutos e legumes), de origem animal, e em alimentos confecionados e congelados (Ooi e Lorber, 2005). *L. monocytogenes* tem também sido isolada de instalações e equipamentos de indústrias alimentares (Lunden *et al*., 2002). Esta bactéria pode encontrar-se no estado planctónico ou formando biofilmes (Adrião *et al*., 2008).

As listerioses são infeções que resultam da ingestão de alimentos contendo *L. monocytogenes* e podem manifestar-se em duas formas clínicas: uma invasiva e outra não invasiva. A forma não invasiva caracteriza-se por uma gastroenterite febril com sintomas semelhantes a uma constipação. A forma de listeriose invasiva, conduz a infeções sistémicas tais como a septicémia e infeções do sistema nervoso central (meningite e encefalite) e afeta fundamentalmente, pacientes de risco (Freitag *et al*., 2009). Neste grupo destacam-se as grávidas, neonatos, idosos e indivíduos portadores de doenças crónicas e imuno-comprometidos (pacientes com cancro, pacientes portadores de VIH e transplantados) (Lecuit *et al*., 2005). A listeriose pode também estar associada a peritonites, endocardites e artrites sépticas (Doganay *et al*., 2003). As células de *L. monocytogenes* têm capacidade de atravessar a placenta e infetar o feto levando a abortos (Drevets e Bronze, 2009).

A patogenicidade de *L. monocytogenes* tem sido explicada pela sua capacidade de transpor as diversas barreiras de proteção endoteliais e epidérmicas e de se tornar um microrganismo patogénico intracelular facultativo de macrófagos, fibroblastos e células epiteliais (Pizarro-Cerdá *et al*., 2012). Assim, após a ingestão a bactéria poderá invadir as células do epitélio intestinal e disseminar-se pela corrente sanguínea e linfática, podendo atingir o fígado e o baço, onde se poderá multiplicar, pois possui mecanismos que lhe permitem reproduzir-se no interior de macrófagos e neutrófilos. Estes mecanismos incluem um conjunto de fatores de virulência que estão subjacentes à sua capacidade de infeção de células eucariotas, dos quais se destacam as internalinas A e B, a listeriolisina O, as fosfolipases C, a proteína Act A, a proteína P60 e o factor PrfA (Freitag *et al*., 2009; Pizarro-Cerdá *et al*., 2012). A internalização de *L. monocytogenes* por células eucariotas é desencadeada pelas proteínas de superfície internalinas A (InlA) e B (InlB), que conferem à bactéria a capacidade de invadir as células do hospedeiro (não fagocíticas), internalizando-se por fagocitose (Fig. 1.2).

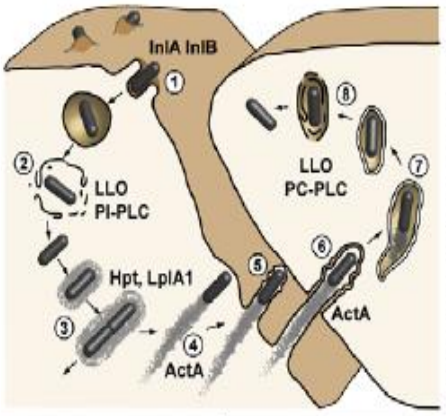

Fig. 1.2. Mecanismo de infeção de *L. monocytogenes* (Cossart e Toledo-Arana, 2008). Legenda: LLO – listeriolisina O; ln1A e ln1B – proteínas de invasão (internalinas); ActA – proteína de mobilidade; PI-PLC – Fosfolipase Fosfatidilinositol-específica; PC-PLC – Fosfolipase Fosfatidilcolina-específica; LplA1 – ligase; Hpt – permease.

A bactéria fica aprisionada num vacúolo fagocítico do qual se escapa, devido à atividade da citolisina listeriolisina O (enzima que induz a formação de poros nas membranas dos fagossomas) (Dewamitta *et al*., 2010) e das fosfolipases C (enzimas que induzem a degradação dos fosfolípidos das membranas dos fagossomas) (Pizarro-Cerdá *et al*., 2012), ficando livre no citoplasma. Aqui as bactérias iniciam a sua multiplicação e a produção e polimerização de uma proteína de superfície, a actina (ActA). Os filamentos de ActA organizam-se num dos pólos da bactéria dando origem a uma "cauda" que contribui para movimentar as bactérias para células adjacentes. Os genes que codificam o conjunto de proteínas que constituem os fatores de virulência descritos anteriormente são regulados pelo ativador de transcrição PrfA (Freitag *et al*., 2009).

Estes genes são fracamente expressos fora do hospedeiro mas fortemente induzidos durante a infeção intracelular (Moors *et al*., 1999).

As listerioses humanas têm uma incidência baixa mas uma elevada taxa de mortalidade pelo que constituem um risco severo para a saúde pública. De acordo com o relatório da EFSA (EFSA, 2014) observou-se, na Europa, um aumento de 10,5 % de casos de listerioses relativamente a 2011, tendo-se registado 1642 casos e uma taxa de mortalidade de 17,8 %. Nos Estados Unidos da América, o Center for Disease Control and Prevention (CDC, 2013c) reportou uma taxa de mortalidade por listeriose, entre 2009 e 2011, de 21%.

Na Europa, a maioria dos surtos epidémicos de listeriose conhecidos resultam da ingestão de alimentos derivados de leite, nomeadamente queijos de pasta mole, e de alimentos prontos a ingerir (sandes, tartes salgadas e produtos processados de carne) (EFSA, 2014). No entanto, em 2011 foi descrito um surto de listeriose nos EUA (CDC, 2011c) devido à ingestão de melão Cantaloupe.

De acordo com os critérios microbiológicos da União Europeia (Regulamentos CE 2073/2005 e 1441/2007), *L. monocytogenes* deve estar ausente em alimentos prontos a ingerir (25 g) destinados a crianças. Esta bactéria não deve ser enumerada em níveis que excedam 100 ufc/g durante o tempo de prateleira de outros alimentos prontos a comer. Nos alimentos que suportam o crescimento de *L. monocytogenes*, a bactéria não deve ser detetada no momento em que o alimento sai da fábrica, a não ser que o produtor garanta que a população microbiana não aumenta para níveis que ultrapassem as 100 ufc/g durante o tempo de prateleira. De acordo com os critérios microbiológicos, os valores mais elevados de *L. monocytogenes* registados que não se encontravam em conformidade com os referidos critérios, nos pontos de venda, foram encontrados nos alimentos prontos a ingerir produzidos com pescado (0,5 %) e com carne (0,4 %). No que diz respeito aos alimentos analisados no final do processamento, os valores mais elevados de não conformidade com os critérios foram observados nos alimentos prontos a ingerir derivados de pescado (8,0 %) seguidos dos queijos (7,2 %). As FCF são alimentos prontos a consumir, não são submetidos a tratamentos térmicos antes do seu consumo e são conservados a temperaturas de cerca de 4 °C, condições em que *L. monocytogenes* sobrevive e tem capacidade para se multiplicar e aumentar a sua população, durante o tempo de vida útil destes alimentos o que os torna particularmente preocupantes enquanto possíveis veículos desta bactéria. A capacidade de

L. monocytogenes crescer nos alimentos a uma determinada temperatura depende de vários fatores, tais como o pH, a atividade da água e a composição do alimento e varia nos vários alimentos, pelo que deverá ser estudada caso a caso (EFSA, 2014).

No que diz respeito às frutas, vários estudos têm demostrado a capacidade de crescimento de *L. monocytogenes* em melão Cantaloupe e Honeydew e melancia minimamente processados (Danyluk *et al*., 2014). O modelo de crescimento desenvolvido por estes autores, previu um aumento de 4 log ufc/g em 15 dias a 5 °C e de 1 log ufc/g em 6 dias a 4 °C das contagens de *L. monocytogenes* nas frutas estudadas. Fang *et al*. (2013) estudaram o comportamento da população de *L. monocytogenes* inoculada em amostras de melão Cantaloupe minimamente processado armazenadas a 4, 8, 16, 20, 30, 40 e 43 °C durante 5 dias e observaram crescimento a todas as temperaturas.

Para além dos aspetos referidos uma das características particularmente preocupantes de *L. monocytogenes* é a sua capacidade de tolerar ambientes ácidos tais como os encontrados no estômago e em alguns alimentos.

1.3.4. Fontes de contaminação

Os frutos podem ser contaminados com microrganismos patogénicos em qualquer etapa desde a sua produção no campo até ao momento do consumo. As condições e práticas ocorridas durante as fases de pré-colheita, colheita e pós-colheita, afetam a presença de microrganismos em frutas e legumes minimamente processados e, consequentemente, a sua qualidade microbiológica e segurança alimentar.

A sobrevivência e o crescimento dos microrganismos na fruta são afetados por variados fatores intrínsecos e extrínsecos. Entre os fatores intrínsecos contam-se o pH, a capacidade tamponizante, o conteúdo em nutrientes, a atividade da água (a_W), o potencial redox, a textura dos tecidos e a presença de compostos antimicrobianos. Os fatores extrínsecos mais relevantes são a temperatura, a humidade e a composição da atmosfera gasosa. No entanto, as condições dependentes das operações de processamento selecionadas podem alterar os fatores intrínsecos, contribuindo para alterar a população microbiana. Por outo lado, a sobrevivência e o crescimento dos microrganismos nos frutos está também dependente da sua atividade fisiológica (viabilidade, taxa específica de crescimento) e capacidade de adaptação a condições de stresse e do seu comportamento em populações mistas (competição, antagonismo e sinergismo) (Beuchat, 2002).

Pré-colheita e colheita

A natureza da microbiota dos frutos, durante a fase de pré-colheita, depende da localização geográfica, da precipitação, da temperatura, do vento, da presença de determinados vetores (insetos, nematodos) e das práticas de agricultura implementadas.

O solo é o reservatório de uma enorme variedade de microrganismos patogénicos e não patogénicos. As espécies *Bacillus cereus*, *L. monocytogenes* e *C. botulinum* ocorrem naturalmente no solo e matéria vegetal em decomposição e podem estar presentes sobre a superfície de produtos frescos (Guinebretiére *et al*., 2003, Guinebretiére e Nguyen-The, 2003; Thevenot *et al*., 2006). Outros microrganismos patogénicos de origem humana/animal podem também ser encontrados no solo devido à utilização de águas contaminadas na irrigação e preparação de fertilizantes e devido à aplicação de adubos orgânicos não tratados ou ainda, devido à presença de excrementos de origem animal nas áreas de cultivo (Nguyen-The, 2012). A presença desses microrganismos no solo como *Escherichia coli* O157:H7, *Salmonella* spp. e *Campylobacter* spp., depende da sua capacidade de sobreviver neste ambiente, que pode variar entre 30 e 980 dias (Heaton e Jones, 2008).

A existência de microrganismos patogénicos em vegetais resultante da utilização de fertilizantes orgânicos e águas contaminados tem sido descrita por diversos autores. Ibenyassine *et al*. (2007) observaram uma elevada incidência de *E. coli*, *Enterobacter cloacae* e *Klebsiella pneumoniae,* em diversos vegetais irrigados com águas residuais não tratadas. Loncarevic *et al*. (2005) detetaram a presença de *L. monocytogenes* e *E. coli* em alfaces cultivadas organicamente e Melloul *et al*. (2001) encontraram as mesmas estirpes de *Salmonella* nas partes comestíveis dos vegetais e na água não tratada utilizada na rega das plantas. O mesmo estudo revelou que os vegetais crescidos à superfície do solo (alface, salsa) estavam mais contaminados do que aqueles que se desenvolveram acima da superfície (tomate). Noutro trabalho, observou-se que *E. coli* O157:H7 persistiu no solo 154-196 dias após a adição do fertilizante orgânico ou água contaminados tendo sido posteriormente detetada em cebolas e em cenouras após 74 e 168 dias, respetivamente (Islam *et al*., 2005).

Outra fonte possível de contaminação de vegetais frescos com organismos patogénicos é a presença de escoamentos provenientes de pastagens de gado (Muirhead *et al*. 2006). Por outro lado, a contaminação microbiana pode ser intensificada na presença de alguns vetores tais como insetos, nematodes e protozoários. Sela *et al*. (2005) mostraram que a transmissão de *E. coli* em maças pode ocorrer através de *Drosophila melanogaster*. A transferência de *Salmonella* spp. do solo para frutas e legumes através do nematode *Diploscapter* sp., foi observada por Gibbs *et al*. (2005). Além disso, os protozoários presentes nas folhas de vegetais (alface), parecem desempenhar um papel especial na proteção e sobrevivência de microrganismos patogénicos de origem alimentar (*E. coli* e *S. enterica*) (Gourabathini *et al*., 2008). O vento constitui outro meio de transferência de poeiras contaminadas com esporos de bactérias ou de fungos (Heard, 2002).

A colonização de sementes por microrganismos patogénicos endófitos é também um aspeto que poderá facilitar a transmissão desses micróbios para gerações seguintes e a prevalência das contaminações nos vegetais (Tyler e Triplett, 2008).

Os principais fatores responsáveis pela contaminação por microrganismos patogénicos de produtos frescos nas fases de pré-colheita estão representados na Figura 1.3.

Os alimentos de origem vegetal podem também ser contaminados por diversos microrganismos durante as etapas de colheita (recolha, seleção, embalagem e transporte), por contaminação cruzada a partir de material fecal, dos equipamentos e recipientes de colheita, dos contentores e dos veículos de transporte (Heaton e Jones, 2008). Nestas fases, a eventual presença de animais selvagens e domésticos, aerossóis, e a utilização de gelo ou água contaminados contribuirão para aumentar os níveis de contaminação microbiana de frutas e produtos hortícolas (Beuchat, 2002, Johnston *et al*., 2005). Além destes aspetos, a manipulação humana pouco cuidada pode causar lesões nos frutos que levam à libertação de nutrientes e promovem a sobrevivência e o crescimento microbiano constituindo portas de entrada para microrganismos patogénicos e de degradação. Nesta fase, a saúde e higiene pessoais e a observação de boas práticas de manuseamento têm de ser tomadas em consideração para reduzir o potencial de contaminação por microrganismos patogénicos neste tipo de alimentos.

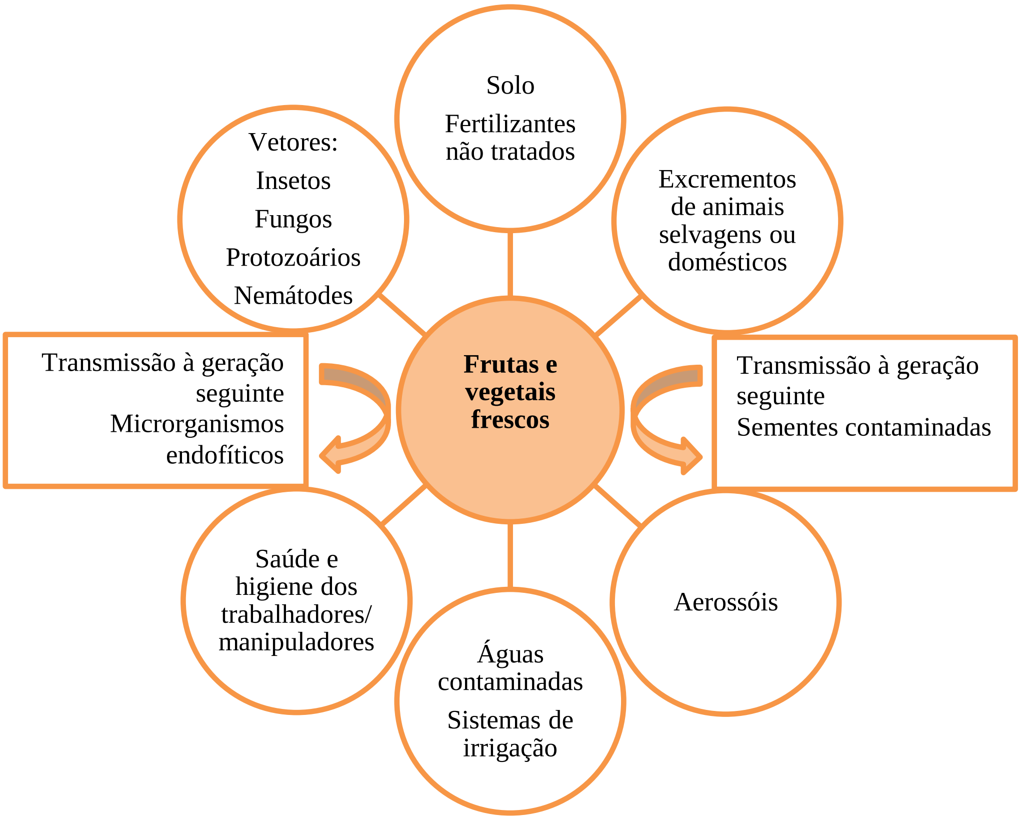

Fig. 1.3. Principais fatores de contaminação de frutas e vegetais frescos por microrganismos patogénicos na fase de pré-colheita (Adaptado de Quintas, 2011).

Os contentores, recipientes e todos os equipamentos utilizados na colheita e transporte podem tornar-se uma fonte adicional de contaminação se não forem adequadamente higienizados (Heaton e Jones, 2008). Este aspeto é particularmente relevante sempre que ocorre formação de biofilmes os quais contribuem para aumentar a capacidade de sobrevivência e de crescimento dos microrganismos e conferir proteção contra os agentes de higienização e desinfetantes. Em qualquer etapa da colheita, se houver contacto dos microrganismos dos biofilmes presentes nas superfícies dos recipientes/equipamentos com os frutos, pode ocorrer contaminação (Beuchat, 2002; Corbo *et al*., 2010).

Finalmente, a microbiota nos frutos, antes da transformação é influenciada por condições tais como a temperatura, a humidade e o tempo de transporte. Após a colheita, os frutos continuam a respirar e a utilizar os açúcares e os ácidos orgânicos disponíveis produzindo dióxido de carbono e etileno, o que pode conduzir à sua rápida senescência (Corbo *et al*., 2010).

A produção de alimentos microbiologicamente seguros, incluindo os frutos, implica a adoção de boas práticas agrícolas e boas condições higio-sanitárias durante as fases de pré-colheita e colheita, de forma a reduzir o potencial de contaminação por microrganismos patogénicos.

Pós-colheita

Durante as diversas etapas da pós-colheita podem surgir condições que contribuem para alterar a microbiota e aumentar a sua população nos frutos minimamente processados. A microbiota da matéria-prima utilizada, a higiene das superfícies dos materiais e equipamentos de processamento e o ambiente fabril, bem como a higiene dos manipuladores de alimentos, são fatores determinantes da qualidade microbiológica do produto final. Outras fontes de contaminação referidas anteriormente, são também relevantes nas etapas de pós-colheita tais como, a presença de animais domésticos e selvagens, insetos, poeiras ambientais, a produção de aerossóis e a qualidade da água (lavagem e enxaguamento) e gelo, entre outros (Abadias *et al*., 2008; Johnston, *et al*., 2005; Lehto *et al*., 2011).

Como já referido anteriormente, os FMP são normalmente submetidos a tratamentos de lavagem e enxaguamento para remover a poeira e detritos, reduzir os níveis de microrganismos, de forma a aumentar a qualidade dos produtos e o seu tempo de vida útil. A água e o processo de secagem dos frutos são fatores que podem levar à contaminação ou causar danos nos tecidos. Johnston *et al*. (2005) observaram um aumento do nível de coliformes totais em salsa e coentros depois da etapa de lavagem. Gagliard *et al*. (2003), constataram o mesmo em melão Cantaloupe, tendo registado um aumento da população de coliformes totais na superfície da fruta após a lavagem. Estes trabalhos demonstraram que é fundamental adotar estratégias para manter a qualidade da água de lavagem e enxaguamento, usando água potável e desinfetantes adequados. A temperatura da água deve também ser controlada e a imersão dos frutos deve ser feita com cautela. Se a diferença de temperatura entre o fruto e a solução de lavagem for muito elevada pode haver infiltração da solução (e de microrganismos) no fruto através de aberturas na pele, no tecido vascular do pedúnculo, das lenticelas, dos estomas, das feridas ou outras disrupções físicas (Parish *et al*., 2003). Posteriormente, o processo de eliminação do excesso de água deve ser adequado a cada fruto de maneira a evitar danos físicos e garantir a remoção completa da água da superfície dos frutos A presença de gotículas de água nas frutas e vegetais processados ou não processados permitirá a multiplicação de microrganismos e a sua mobilidade nos casos dos microrganismos móveis (patogénicos ou degradadores da fruta) (Soliva-Fortuny e Martín-Belloso, 2003).

Durante o processamento, operações como o descasque e o corte, quebram a barreira de proteção do fruto conferida pela casca/pele, aumentando assim a disponibilidade de açúcares e de outros nutrientes, o que promove um ambiente favorável à sobrevivência de microrganismos contaminantes e ao seu rápido crescimento (Corbo *et al*., 2010; Harris *et al*., 2003; Oms-Oliu *et al*., 2010; Pasha *et al*., 2014). Estas operações apresentam o risco de transferência de microrganismos patogénicos da superfície do fruto intacto para os seus tecidos internos (Fang *et al*., 2013; Harris *et al*., 2003; Olaimat e Holley, 2012). Ukuku e Sapers (2001) observaram que células de *Salmonella* presentes na casca foram transferidas para a superfície de fatias de melão Cantaloupe.

As operações de descasque e redução de dimensões apresentam ainda o risco de contaminação e recontaminação devido ao contacto de equipamentos contaminados com os alimentos. Um estudo conduzido em unidades industriais de processamento de vegetais minimamente processados revelou elevados níveis de microrganismos aeróbios totais em todas as superfícies de contacto com alimentos, nomeadamente nos equipamentos de descasque, facas, tábuas de corte, entre outros (Lehto *et al*., 2011). Os mesmos autores reportaram valores elevados de contagens de bactérias da família *Enterobacteriaceae* (β-Glucuronidase positiva) nas cortadoras e nas tábuas de corte.

O embalamento e o armazenamento são etapas relevantes que podem proporcionar condições de contaminação e crescimento de microrganismos em frutas e legumes. Prazak *et al*. (2002) observaram a sobrevivência e a proliferação de *Listeria* spp. nas correias transportadoras, durante o processo de embalagem, podendo ocorrer a contaminação cruzada entre estas superfícies e os frutos. Os mesmos autores reportaram um aumento da carga microbiana em melão Cantaloupe durante o processo de embalagem. Por outro lado, Lehto *et al*. (2011) detetaram valores de microrganismos aeróbios totais elevados na atmosfera das zonas de armazenamento, processamento e embalamento.

A obtenção de frutas minimamente processadas microbiologicamente seguras, implica a adoção de boas práticas de fabrico e boas condições higio-sanitárias durante todas as etapas de pós-colheita devendo assegurar-se um conjunto de medidas de controlo, com vista a produzir alimentos microbiologicamente seguros (Tabela 1.7).

Tabela 1.7. Medidas preventivas para reduzir agentes patogénicos alimentares durante as etapas de pós-colheita de frutos frescos (Quintas, 2011).

Utilização de água de boa qualidade na: Lavagem Enxaguagem Desinfeção
Evitar abusos de temperatura em: Armazenamento Transporte Distribuição Exposição/Marketing Consumidor (casa)
Evitar a humidade no ambiente de processamento e sobre a superfície de frutos: Evitar a condensação nas embalagens Fornecer uma secagem adequada após a lavagem e enxaguamento
Utilizar equipamento de corte adequado e bem afiado para: Minimizar a destruição dos tecidos
Evitar as contaminações cruzadas mediante: Separação de frutos processados de frutos não processados Separação de alimentos de origem animal de alimentos de origem vegetal
Aplicar programas de limpeza e desinfeção de superfícies de contato com alimentos e equipamentos com a frequência adequada Instalações Equipamentos Evitar a formação de biofilmes Evitar a formação de aerossóis
Boas práticas de fabrico Prevenir a contaminação fecal Higiene pessoal rigorosa Providenciar a educação para manipuladores de alimentos
Ter consciência de que os microrganismos se adaptam e evoluem Os microrganismos podem sofrer mutações Os microrganismos podem adaptar-se ao stresse e aos biocidas Os microrganismos podem adquirir virulência

1.4. Objetivos

As frutas minimamente processadas, são alimentos crus prontos a consumir que podem ser contaminados em qualquer fase do seu processamento e a única operação de redução da população microbiana a que são submetidos é a desinfeção. O método de desinfeção mais utilizado, o cloro, apresenta diversas limitações e riscos para a saúde humana e ambiental, pelo que é de extrema importância estudar a aplicação de outros métodos de redução da população microbiana nos frutos.

Os objetivos gerais deste trabalho foram estudar a qualidade microbiológica de fruta minimamente processada (FMP) comercializada em Portugal (Algarve) e estudar a aplicação de tecnologias de desinfeção alternativas ao cloro, com vista ao aumento da segurança microbiológica destes alimentos.

Para alcançar os referidos objetivos gerais, estabeleceram-se os seguintes objetivos específicos:

1. Avaliar a qualidade microbiológica (microrganismos aeróbios mesófilos, psicrotróficos, bactérias ácido-lácticas, fungos e coliformes) de FMP disponível no mercado português, em particular no Algarve, e detetar a presença de microrganismos patogénicos de origem alimentar (*Escherichia coli*, *Salmonella* spp., *Listeria monocytogenes*, *Cronobacter sakazakii* e *Staphylococcus aureus*).
2. Estudar a sobrevivência e a capacidade de crescimento de *E. coli*, *S. enterica* e *Listeria* spp. em pera 'Rocha', a diferentes temperaturas.
3. Estudar o efeito de um método químico de desinfeção, a água electrolisada (ácida e neutra), na redução das populações de *E. coli*, *S. enterica* e *Listeria* spp., previamente inoculadas em pera 'Rocha'.
4. Estudar o efeito de um método físico de desinfeção, a iluminação UV-C, na redução das populações de *E. coli*, *S. enterica* e *Listeria* spp., previamente inoculadas em pera 'Rocha' e maçã 'Golden Delicious'.
5. Determinar o efeito dos tratamentos de água electrolisada e iluminação UV-C nos parâmetros de qualidade da FMP.

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Capítulo II

Evaluation of microbial quality and yeast diversity in fresh-cut apple

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Evaluation of microbial quality and yeast diversity in fresh-cut apple

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ABSTRACT

The present work's aim was to study the microbial quality of minimally processed apples commercialized in Portugal. Sixty eight samples of fresh-cut apple were analyzed before their best-before date in 2011 and 2012 for aerobic mesophilic and psychrotrophic microorganisms, total coliforms, lactic-acid bacteria (LAB), coagulase-positive staphylococci and fungi. The parameters of food safety studied were *Cronobacter sakazakii*, *Salmonella* spp. and *Listeria* sp. Samples were analyzed according to standard methodologies and using Chromocult Agar for coliforms and *Escherichia coli*. The yeasts were identified by restriction analysis of the ITS-5.8S rDNA-region and 26S rDNA partial sequencing.

The mesophilic and psychrotrophic microorganisms ranged from 3.3 to 8.9 and from 4.9 to 8.4 log CFU/g, respectively. Coliforms were detected in all the samples and staphylococci in 5.8% of them. LAB numbers varied from 2.8 to 8.7 and fungi (yeast and molds) from 3.6 to 7.1 log CFU/g. The most common yeasts were *Candida sake* and *Pichia fermentans* followed by *Hanseniaspora* spp., *Candida* spp., *Meyerozyma guilliermondii*, *Metschnikowia pulcherrima*, *Cryptococcus* spp. and the psychrotrophic *Cystofilobasidium infirmominiatum*. Foodborne bacteria and opportunistic pathogenic yeasts were not detected in the apples studied. The results obtained respected the European Commission regulation regarding criteria of food hygiene and safety.

1. Introduction

Fresh products, like fruits and vegetables, are important components of a balanced diet and a healthy life style and their consumption is being encouraged by several government health authorities all over the world. These trends stimulated the growing demand for "quick" and convenient fresh food products and the rapid growth of the fresh-cut fruits and vegetables' industry. Minimally processed foods offer many advantages, as they reduce the meal preparation time and increase access to food that retains high nutritional and sensory quality.

Minimally processed fruits and vegetables are fresh, raw fruits or vegetables processed in order to supply a ready-to-eat or ready-to-use food product. The processing of the foods may include cutting, peeling, slicing, shredding, trimming, washing and disinfecting. Products are packed and refrigerated becoming ready-to-eat items that are very attractive to consumers looking for healthy and convenient meals. This type of food is characterized by the presence of non-sterile cut surfaces with damaged tissues where active metabolism may occur (Berger et al., 2010; Francis et al., 2012; Olaimat and Holley, 2012). Fresh-cut fruits are susceptible to microbial contamination in any phase of the production or distribution, due to destruction of natural protective barriers and their high water and nutrient contents. Additionally, they are neither heat treated nor contain added preservatives. As a result, they may be a vehicle for microbial pathogens causing health problems. Fruits may contain various microorganisms that are naturally present in nature or acquired/gained during harvest, processing operations or even during the handling by workers or consumers. If the initial load of microorganisms is high and/or the preparing operations are inadequate, some microorganisms will survive and subsequently will grow and cause spoilage and possibly illness if

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the surviving microorganisms are virulent (Beuchat, 2002).

Although an extensive range of minimally processed vegetables have been offered to consumers for many years, the market for minimally processed fruits is economically less important, even though it has an interesting potential for growth, as fruits are relevant sources of vitamins, minerals, sugars and organic acids. In fact, fresh-cut fruit is being offered as an alternative to whole fruits in restaurants, supermarkets and in caterings (e.g. airline travel). Fresh-cut apples were the first kind of fresh-cut fruit to appear in the Portuguese market and presently are available in restaurants and supermarkets, thus contributing to a healthier dessert alternative to sweets, especially for children.

Teixidó et al. (1999) studied the microbial aspects of "Golden delicious" apples in the orchard and found that the main microbiota were fungi (*Cladosporium*, *Alternaria* and yeasts). After this work, Abadias et al. (2006) analyzed apples of the same variety throughout the production and shelf-life regarding their contamination with bacteria of the family *Enterobateriaceae* and did not find *Salmonella* or pathogenic *Escherichia coli*. However, low levels of other enterobacteria (*Pantoea*, *Citrobacter*, *Enterobacter*, *Klebsiella* and *Escherichia*) were identified. Numerous studies (e.g. Abadias et al., 2009; Alegre et al., 2010a,b) have shown that different pathogenic bacteria, like *E. coli*, *Salmonella* spp., and *Listeria monocytogenes* can survive and grow in fresh fruit tissues, such as apples and peaches, stored with no refrigeration or at temperatures of 10 °C or higher. Under refrigeration conditions (5 °C) *E. coli* was able to survive in apple wounds, flesh, peel and juice (Abadias et al., 2009).

Therefore, fresh-cut fruit can be a vehicle for the transmission of foodborne pathogens such as *E. coli*, *Salmonella* spp. and *L. monocytogenes* (Sivapalasingam et al., 2004). For example, non-pasteurized apple cider (Beuchat, 2002) and strawberries (FDA, 2011) were responsible for outbreaks of a foodborne illness due to *E. coli* O157:H7; cantaloupe (CDC 2011) was implicated in outbreaks due to *L. monocytogenes*; fruit salad, cantaloupe, papaya (CDC, 2011) and watermelon (Beuchat, 2002) were responsible for salmonellosis outbreaks.

Fungi, especially yeasts, are another major concern in fresh-cut fruits as they can be responsible for spoilage by changing color, producing gas, off-flavors and souring (Loureiro and Querol, 1999; Tournas et al., 2006). The surface of whole fruits carries yeasts populations (Teixidó et al., 1999) that can cross-contaminate the fresh-cut fruit during processing. Several genera of yeasts such as *Candida*, *Cryptococcus*, *Debaryomyces*, *Kloeckera*, *Kluyveromyces*, *Pichia*, *Rhodotorula*, *Saccharomyces* and *Zygosaccharomyces* have being referred to be present in fresh fruits (Ruiz-Cruz et al., 2010; Tournas et al., 2006).

Despite the microbiological problems (safety and spoilage) associated with fresh-cut produce, little data exists on evaluating the microbial quality of fresh-cut fruit available in the Portuguese commerce. To this end, the main aim of the present work was to study the microbiological quality of minimally-processed apples commercialized in the south of Portugal, including the detection of some bacterial foodborne pathogens (*Salmonella* spp., *Cronobacter sakazakii* and *L. monocytogenes*). Additionally, changes in the microbiota were studied in packages sampled prior to and after their best-before date. Finally, another objective of this work was to characterize the yeast population present in the fresh-cut apples studied due to the importance of these microorganisms as spoilers in low pH food.

2. Methods

2.1. Samples

During 2011 (21 samples) and 2012 (47 samples), a total of 68 samples of fresh-cut apples were purchased in restaurants and supermarkets in southern Portugal before their best before date. Samples were obtained throughout the year and consisted of individual packs of 70 g–100 g of apple slices that were transported to the laboratory in a cooler box and analyzed on the day of purchase. The 2011 samples were collected from mid-September until the end of November. The 2012 samples were bought in two periods; the first period, from May to the end of July (23 samples) and the second period, from mid-September till the beginning of December (24 samples). Two samples were studied per week. Data related to the samples were recorded, including date and place of purchase, lot number and expiry date.

2.2. Microbial analysis

Samples were analyzed, respecting the aseptic manipulation, according to standard (ISO) methodologies, summarized in Supplemental Table S1, and using Chromocult Agar (Merck, Darmstadt, Germany) for total coliform and *E. coli* (González et al., 2003) and Brilliance *Enterobacter sakazakii* chromogenic DFI (Druggan-Forsythe-Iversen, Oxoid, Basingstoke, England) for *C. sakazakii* (Chap et al., 2009).

Twenty five grams of each sample were diluted in 225 mL of buffered peptone water (BPW, Oxoid) and homogenized in a Stomacher (Model 400 Circulator, Seward, Norfolk, England) for 2 min at normal speed. From this suspension the enumeration of mesophilic (AM) and psychrotrophic microorganisms (PM), lactic acid bacteria (LAB), total coliform (TC) and *E. coli*, yeasts and molds (YM) and staphylococci (STAPH) was performed. The remaining suspension was incubated at 37 °C for 18 h for the detection of *Salmonella* spp. and *C. sakazakii*. The detection of total coliform and *E. coli* was performed by spread plating aliquots of the serial dilutions in Chromocult agar (Merck). To detect *C. sakazakii*, 10 mL of the pre-enrichment suspension on buffered peptone water was inoculated in EEbroth (Scharlau, Barcelona, Spain). After a 24 h incubation at 37 °C, an aliquot was inoculated on Brilliance *E. sakazakii* chromogenic DFI (Oxoid). According to the methodology, another portion of 25 g of the sample was homogenized in 225 mL Half-Fraser broth (Oxoid) and incubated at 30 °C for 24 h for the detection of *L. monocytogenes*.

In an extra set of 12 samples of fresh-cut apples, in addition to the 68 analyzed, six were analyzed 5–6 days prior to their best-before date for mesophilic and psychrotrophic microorganisms, total coliforms, LAB and fungi. The remaining samples were stored at 4 °C and analyzed 10–15 days after their expiration date, when visible spoilage/alterations were detectable for the same microbiological parameters.

2.3. Foodborne pathogens confirmation

Salmonella spp., *L. monocytogenes* and *C. sakazakii* presumptive isolates, obtained after the selective plating, were streaked onto tryptic soy agar TSA (Scharlau, Barcelona, Spain) until purification of the colonies. Single colonies were Gram stained and tested for cytochrome c oxidase activity and catalase activity. The confirmation of the pathogenic bacteria was performed using PCR with specific genus and species primers. Pure colonies of presumptive *Salmonella* spp. and *C. sakazakii* were sub-cultured in Brain Heart Infusion (Scharlau) and *L. monocytogenes* in TSB (Biokar Diagnostics, Beauvais, France) with yeast extract (0.6%) for further DNA extraction. Gram positive bacteria DNA extraction was performed using the guanidine thiocyanate method (adapted from Pitcher et al., 1989) and Gram negative bacteria DNA extraction was performed with the boiling method (Sambrook et al., 1989). All the PCR reactions were performed in volumes of 70 µl containing

50–500 ng DNA, 0.5 µM each primer, 10 µM deoxynucleotides (Promega, Madison, USA), 1.5 mM $MgCl_2$ and 1 unit DNA polymerase (Promega). Amplifications were performed in a thermocycler (My Cycler thermal cycler, BioRad, Hempstead, United Kingdom).

2.3.1. *Listeria spp. and L. monocytogenes*

For *Listeria* genus-specific and *L. monocytogenes* species-specific identifications by PCR of the presumptive isolates from 4 samples of fresh-cut apple, two different primer combinations derived from the iap gene, that encodes for the protein p60 (invasion associate protein), were used: pair UnilisA (5′-GCTACAGCTGGGATTGCGGT-3′) and Lis1B (5′-TTATACGCGACCGAAGCCAA-3′) (Bubert et al., 1997). For the species *L. monocytogenes*, the primer pair MonoA (5′-CAAACTGCTAACACAGCACT-3′) and MonoB (5′-GCACTT-GAATTGCTGTTATTG-3′) was used (Bubert et al., 1997). The DNA amplification started with an initial denaturation phase at 94 °C for 3 min, followed by 30 cycles, each at 94 °C for 1 min, 56 °C for 45 s and 72 °C for 45 s. An elongation phase was performed at 72 °C for 5 min. The *Listeria* genus specific amplification product has 1.4 kb and the *L. monocytogenes* specific amplification DNA product has approximately 400 bp (Bubert et al., 1997). Fragment sizes were estimated by comparison against a DNA ladder (NZYDNA Ladder 5, NZYTech, Lisbon, Portugal).

2.3.2. *Cronobacter spp. and C. sakazakii*

The presumptive identification of *Cronobacter* spp. isolates, obtained from 2 samples, was evaluated through the amplification of the gene putatively encoding the α-glucosidase (*gluA*), according to Lehner et al. (2006). The primers were: EsAgf: 5′-TGA AAG CAA TCG ACA AGA AG-3′ and EsAgr: 5′-ACT CAT TAC CCC TCC TGA TG-3′. Thermal cycling was carried out by using an initial denaturation step of 94 °C for 2 min, followed by 29 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 60 s and extension at 72 °C for 90 s. Cycling was completed by an elongation step at 72 °C for 5 min. The amplification product generated has 1680 bp.

The species *C. sakazakii* was identified with primer pair Csakf (5′-ACGCCAAGCCTATCTCCGCG-3′) and Csakr (5′-ACGGTTGGCGT-CATCGTG-3′) and PCR conditions used were denaturation at 94 °C for 3 min, followed by 30 cycles of 94 °C for 1 min, annealing at 67 °C for 30 s, and 72 °C for 1 min. Final elongation was performed at 72 °C for 5 min (Stoop et al., 2009). The amplification products generated have about 500 bp. *C. malonaticus* identification was also performed using the primer pair Cmalf (5′-CGTCGTATCTCTGCTCTC-3′) and Cmalr (5′-AGGTTGGTGTTCGCCTGA-3′) (Stoop et al., 2009). PCR conditions were denaturation at 94 °C for 3 min, followed by 30 cycles of 94 °C for 1 min, 60 °C for 30 s, and 72 °C for 30 s. Elongation was at 72 °C for 5 min. The amplification product generated has 251 bp. Fragment sizes were estimated by comparison against a DNA ladder (NZYDNA Ladder 1 and NZYDNA Ladder 5, NZYTech).

2.3.3. *Salmonella spp.*

The presumptive identification of *Salmonella* spp. isolates, obtained from 7 samples, was evaluated through the amplification of the gene located on the pathogenicity island 1 of bacteria belonging to the genus *Salmonella*. This gene, *invA*, encodes proteins of the type III secretion system described as essential for the invasion of epithelial cells by *Salmonella*. The PCR amplifications were performed with primers 139f 5′-GTGAAATTATCGCCACGTTCGGGCAA-3′ and 141r 5′-TCATCGCACCGTCAAAGGAACC-3′ (Rahn et al., 1992). Thermal cycling started with an initial denaturation step of 95 °C for 1 min, followed by 38 cycles of 30 s at 95 °C, 30 s at 64 °C, 30 s at 72 °C and a final extension of 4 min at 72 °C. The amplicon generated has 285 bp (Malorny et al., 2003).

All the amplification products were electrophoresed in a 1% agarose gel in TAE buffer and stained with ethidium bromide. Gels were visualized under UV light in a G-Box Syngene-Genesis 10 UV Scanner (Cambridge, United Kingdom). Fragment sizes were estimated by comparison against a DNA ladder (NZYDNA Ladder 5, NZYTech).

2.4. *Yeast isolation and identification*

After the incubation period, colonies were counted and yeast representative colonies were isolated and sub-cultured onto YM agar (yeast extract 3.0 g/l, malt extract 3.0 g/l, peptone, 5.0 g/l, dextrose 10.0 g/l and agar 20 g/l) (Scharlau). Yeast colonies of all morphotypes were selected according to their macro and micro morphology, isolated in proportion to their frequencies and sub-cultured for subsequent identification. The isolated strains were preserved at −80 ± 5 °C using glycerol (20% v/v) as cryoprotectant agent.

Yeast identification was performed by the PCR-RFLP (5.8S-ITS region) method described by Esteve-Zarzoso et al. (1999) and by sequence analysis of the region D1/D2 of the 26S rRNA gene (Kurtzman and Robnett, 1998).

Amplification of the 5.8S-ITS region: was done with the primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′ TCCTCCC GCTTATTGATATGC-3′) (White et al., 1990). DNAs from liquid cultures were extracted according to Querol et al. (1992). PCR reactions and amplifications conditions were performed as described in Santo et al. (2012). PCR products (10 µl) were digested with the restriction enzymes *Cfo*I (Sigma–Aldrich, St. Louis, USA), *Hae*III (Promega, Madison, USA) and *Hinf*I (Promega). The PCR products and their restriction fragments were separated by electrophoresis on 1% and 3% (w/v) agarose gels, respectively, with 1 × TAE buffer. After staining with ethidium bromide gels were visualized under UV light in a G-Box Syngene-Genesis 10 UV Scanner. Fragment sizes were estimated by comparison against a DNA ladder (NZYDNA Ladder V, NZYTech). Yeast isolates were grouped according to their RFLP profiles and compared with the RFLP patterns described in Coton et al. (2006), de Llanos Frutos et al. (2004), Esteve-Zarzoso et al. (1999), Santo et al. (2012) and Valles et al. (2007).

Sequence analysis of the region D1/D2 of the 26S rRNA gene: A representative number of isolates in each PCR-RFLP profile obtained were treated to perform sequence analysis of the domains D1 and D2 of the 26S rRNA gene (In the RFLP profiles containing up to six isolates all the yeasts were sequenced. In the case of RFLP profiles containing between 11 or 12 isolates, 50% of the isolates were sequenced and in the groups containing more than 60 isolates, 20 percent of them were sequenced). PCR amplification of the referred region in the 26S rRNA gene with the primers NL1 (5′ GCATATCAATAAGCGGAGGAAAAG-3′) and NL4 (5′-GGTCCGT GTTTCAAGACGG-3′) (Kurtzman and Robnett, 1998) was performed. The amplification reaction and PCR conditions were described in Santo et al. (2012). Amplified products were sequenced by LGC Genomics (Germany) and sequences were compared to those available in GenBank database at the National Center for Biotechnology Information (NCBI) using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) to be identified by sequence homology with described type species.

2.5. *Data analysis*

Results of microorganisms' abundance are presented as the mean ± standard error of log CFU/g. Combined microorganism's mean abundances (in log CFU/g) in samples prior to best-before date were compared to packages sampled after their expiration date using the Hotelling T^2 test for two independent samples. This is a multivariate extension of the *t* test that tests differences on vectors of mean scores of several dependent variables while addressing the problem of "inflating" Type I error rate that would arise when making a series of *t* tests (Hair et al., 1998).

3. Results and discussion

3.1. Microbial quality

The microbial quality of fresh-cut apple samples studied is presented in Table 1. The values of AM and PM ranged from 3.3 to 8.9 log CFU/g and 4.9 to 8.4 log CFU/g, respectively. Only 3.0% of the samples had AM counts inferior to 10^5 CFU/g and 59.7% of samples had counts between 10^7 and 10^8 CFU/g. Regarding PM 62.9% of samples had counts between 10^7 and 10^8 CFU/g. These values are higher than those described in Abadias et al. (2008) in fresh-cut fruit samples that varied between 2.0 and 7.1 log CFU/g for AM and ranged from 1.7 to 7.1 log CFU/g for PM. Viswanathan and Kaur (2001) also found high counts of AM in fresh-cut pineapple and watermelon with values ranging from 6.9 to 8.2 log CFU/g and 6.9 to 8.0 log CFU/g respectively. On the other hand, Seow et al. (2012) obtained values of AM counts in whole apples of 2.1–5.1 log CFU/g and inferior to 5.1 log CFU/g in the case of PM numbers. However, the microbial counts in whole fruit are lower than in fresh-cut fruit due to the fact that the processing operations are potential sources of contamination (Abadias et al., 2008).

Lactic acid bacteria (LAB) counts ranged between 2.8 and 8.7 log CFU/g. This is in contrast with the lower values obtained by Abadias et al. (2008), 1.7 and 4.8 log CFU/g. Although LAB may include spoilage microorganisms, some recent studies report their use as a biological control agent in fresh-cut fruit. In fact, LAB produce lactic acid, through homolactic or heterolactic fermentative metabolism, and bacteriocins that inhibit the growth of some accompanying microbiota (Alegre et al., 2011).

Regarding the total coliform (TC) counts, the levels found in the samples analyzed herein were between 1.8 and 7.6 log CFU/g and *E. coli* was not detected. According to the regulation of the European Commission (EC, 2005, EC, 2007), the apple samples fulfilled the microbiological criteria of food hygiene, as *E. coli* (hygienic criterion for the manufacturing process) was not detected. In the study by Viswanathan and Kaur (2001), total coliforms ranged from 4.3 to 7.9 log CFU/g in pineapples and between 4.5 and 6.9 log CFU/g in watermelon samples. In the study conducted by Abadias et al. (2008), maximum numbers of Enterobacteriaceae of 4.8 log CFU/g were reported. According to Knittel et al. (1977), coliforms are considered a natural occurrence on produce and their importance as faecal indicators is limited as some species may be of non-fecal origin. Doyle and Erickson (2006) concluded that *E. coli* was a better indicator of fecal contamination than coliforms but more recent genetic information, collected by Luo et al. (2011) may have weaken that conclusion. Moreover, *E. coli* was not detected, even though an increase of *E. coli* population has been reported in cantaloupe after processing steps (Johnston et al., 2006).

Table 1
Results of mesophilic microorganisms (AM), psychrotrophic microorganisms (PM), lactic acid bacteria (LAB), total coliforms (TC) and yeasts and molds (YM) on apple samples.

	Percentage of samples in the indicated interval						
	<10^5	10^5–10^6	10^6–10^7	10^7–10^8	>10^8	Range[a]	Mean[b]
AM	3.0	3.0	25.4	59.7	9.0	3.3–8.9	7.7
PM	1.6	3.2	24.2	62.9	8.1	4.9–8.4	7.6
LAB	1.5	21.2	39.4	33.3	4.5	2.8–8.7	7.4
TC	10.4	29.9	46.3	13.4	0.0	1.8–7.6	6.6
YM	10.6	45.5	40.9	3.0	0.0	3.6–7.1	6.2

[a] Range in CFU/g per gram of apple.
[b] Counts are given in terms of log CFU/g.

In the analyzed fresh-cut apple samples the yeasts and molds (YM) were also present in high numbers, ranging from 3.6 to 7.1 log CFU/g but in relatively lower numbers when compared to bacteria. According to the characteristics of the colonies, this microbial population consisted mainly of yeasts. In the case of fresh-cut fruit analyzed by Abadias et al. (2008), the levels of YM recorded were between 1.7 and 4.9 log CFU/g. Moreover, Tournas et al. (2006) observed mean counts higher than 6.0 log CFU/g in fresh cut cantaloupe, pineapple, watermelon and mixed fruit salads, and of 4.4 log CFU/g in cut strawberries.

The microbiological populations (AM, PM, LAB, TC and YM) of fresh-cut apples studied after their best before period (10–15 days at refrigeration temperatures), when degradation signals were visible, showed a small increase in the parameters AM, PM, and LAB, a more pronounced increase in YM, and a slight decrease in TC levels when compared with the results obtained in the samples analyzed before the expiration date. Overall, the group difference across the vectors of mean scores was found to be slightly non-significant (Hotelling $T^2 = 7.41$, $p = 0.0546$). However, the differences in the mean level of molds and yeasts showed statistical significance (*t* test, $p < 0.002$) (Fig. 1).

3.2. Foodborne pathogens

In the "food safety criteria" at the market place (EC, 2005 and EC, 2007), *L. monocytogenes* and *Salmonella* spp. were not detected in the fresh-cut apple studied. These results are in accordance with similar studies performed with fresh produce where the presence of foodborne microorganism is low or inexistent (Abadias et al., 2008; Johnston et al., 2006 and Santos et al., 2012). Additionally, the emergent pathogen *C. sakazakii* was also not detected in the same samples. The presence of *C. sakazakii* in food is a major concern due to infections it may cause in babies and infants, which makes this bacteria a hazard for children (Chap et al., 2009). However, 5.8% of the samples studied presented coagulase-positive staphylococci numbers ranging from <1 log CFU/g to 3.0 log CFU/g of fruit. The contamination of food with this bacterium may result from inadequate handling during processing but its growth and toxin production depend on the temperature conditions.

3.3. Yeast identification

The 236 yeasts isolates obtained from 25 fresh-cut apple

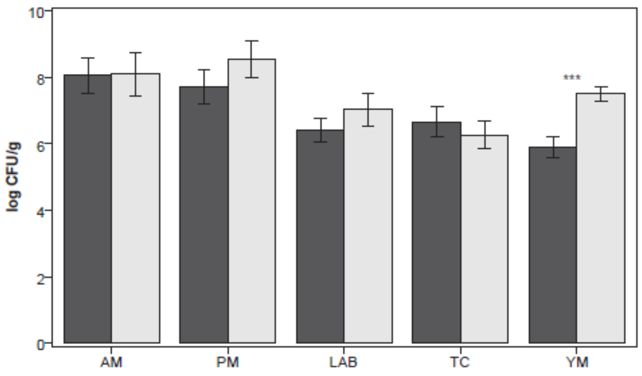

Fig. 1. Comparison of aerobic mesophilic (AM), psychrotrophic microorganisms (PM), lactic acid bacteria (LAB), total coliform (TC) and yeasts and molds (YM) counts (Log CFU/g, mean) in fresh-cut apple before (dark column) and after (white column) the best before date. ***t test, $p < 0.002$.

samples were identified on basis of restriction fragment length polymorphism of the ITS-5.8 rDNA region and of sequencing the 26S rRNA gene' D1/D2 region. The yeast isolates originated PCR products ranging from 400 to 775 bp and their restriction produced 18 different RFLP profiles as shown in Table 2. Isolates in each group ranged from 1 to 113 (Table 2). According to the results, yeast isolates were grouped into 15 species belonging to 10 genera. Profiles I, VI and XV did not originate D1/D2 rDNA sequences identical to any reference species and thus were only associated with genera *Candida*, *Saturnispora*, and *Hanseniaspora*, respectively.

The greatest number of isolates obtained from the minimally processed apples corresponded to the ascomycetous *Candida sake* (profile IV) and *Pichia fermentans* (profile V), which were isolated in frequencies of 47.9 and 25.4%, respectively. *C. sake* is a ubiquitous microorganism, component of the epiphytic microbiota of fruits that has been isolated from the surface of apples and cider musts (Coton et al., 2006) and showed antagonistic activity *in vivo* against a wide spectrum of postharvest pathogens of pears and apples (Viñas et al., 1998; Usall et al., 2000, 2001). *P. fermentans* is a biofilm producer on fruit surfaces and has shown biocontrol activity against brown rot caused by *Monilinia* on apple fruit when inoculated in artificial wounds (Giobbe et al., 2007). Besides *C. sake*, four more species of the genus *Candida* were isolated, *C. incommunis* (5.5%), *C. glaebosa* (0.8%), *C. oleophila* (0.4%) and two isolates of a non-identified species (*Candida* sp.). The species *Hanseniaspora uvarum* (profile XVI) represented 5.5% of the isolates collected from the apples. From the same genera one isolate of *Hanseniaspora osmophila* (profile XVII), two isolates of *Hanseniaspora valbeyensis* (profile XVIII) and one unidentified species (*Hanseniaspora* sp. profile XV) were also collected. The presence of species of the genus *Hanseniaspora* have always been reported in apple/cider musts namely in the early phases of the cider making processes (Coton et al., 2006) constituting major species in the apple juice (Valles et al., 2007). These yeasts, characterized by low fermentative activity, have also been frequently found on the surface of fruit (grapes, strawberry tree fruit), in the first phases of the fermentations (Santo et al., 2012). The genus *Metschnikowia* includes species found commonly in the fruit surfaces and 2.5% of the isolates collected in fresh-cut apples were identified as *M. pulcherrima* (profile II). *Metschnikowia* spp. has been used as a biocontrol agent against postharvest rot caused by *Botrytis cinerea* and other postharvest pathogens in apples, peaches, and grapes (Parafati et al., 2015). It should be noted that the genera *Hanseniaspora* and *Metschnikowia* are the most common non-*Saccharomyces* yeasts found in the beginning of fruits' alcoholic fermentations, including grapes, apples and strawberry tree fruits (Santo et al., 2012; Valles et al., 2007). They have been reported to produce ethyl acetate and acetic acid before and during initial fermentation steps responsible for spoilage of alcoholic beverages (Romano et al., 2003) and may be also related to spoilage of fresh-cut fruit. *Zygotorulaspora fiorentina* (profile IX), a species belonging to a genus frequently related to spoilage of foods, was detected in fresh-cut apples with a percentage of 0.8%. Regarding the basidiomicetous yeasts, *Rhodotorula mucilaginosa* (profile XIV) was isolated in percentage of 1.3% and has been referred to be part of the phyllosphere of plants and isolated from a wide variety of fruits (Janisiewicz et al., 2010; Santo et al., 2012; Trindade et al., 2002). *Cryptococcus carnescens* (profile VII) and *Cryptococcus flavescens* (profile VIII) were identified in relative frequencies of 1.3% and 0.4%. Species of this genus were already isolated from leaves of different Mediterranean plants (Inácio et al., 2005), from Brazilian fruits (Trindade et al., 2002) and nectarines (Janisiewicz et al., 2010). Basidiomicota yeasts belonging to the *Cryptococcus* and *Rhodotorula* are common in fruits and have been reported to be effective as biocontrol agents in nectarines (Janisiewicz et al., 2010). Additionally, some of them have been reported as being able to produce killer toxins (Trindade et al., 2002). The isolates identified as *Cystofilobasidium infirmominiatum* (profile X) were present in a percentage of 1.3% and their behavior as a biological control agent was reported on apples and citrus, against postharvest diseases during storage (Vero et al., 2011). Its presence in frozen vegetables was referred to in Sampaio et al. (2001) and is related to a preference shown by species of the genus *Cystofilobasidium* for low temperatures. According to Tournas et al. (2006) high levels of yeasts may be an indication of high contaminated raw material or lack of adherence to good manufacturing practices during the processing, distribution and selling of the food. In their study, *Pichia* spp., *Rhodotorula* spp., *C. pulcherrima*, *C. lambica*, *C. sake* and *Debaryomyces polymorphus* were the most common yeasts isolated. Lack of hygienic practices during the processing of fresh-cut fruit increases cross contamination and the microbial load on the skinned fruit surfaces. Although some of the yeasts described in the present study are associated to food spoilage, none of the isolates known to be opportunistic pathogens, such as *Candida albicans* and *Cryptococcus*

Table 2
Identification of yeast isolates obtained from the fresh-cut apples through PCR-RFLP and partial sequencing methods (RFLP profiles, PCR products, restriction fragments, isolation percentage and identified species[a]).

RFLP group/ profile	PCR product (bp)	Restriction fragment (bp)			% Isolation	Name[a]
		Cfo I	*Hae* III	*Hinf* I		
I	400	200 + 120 + 80	400	210 + 190	0.9	*Candida* sp.
II	400	205 + 100 + 95	280 + 100	200 + 190	2.5	*Metschnikowia pulcherrima*
III	425	400	425	225 + 200	5.5	*Candida incommunis*
IV	450	250 + 200	450	230 + 220	47.8	*Candida sake*
V	450	170 + 100 + 100 + 80	340 + 80 + 30	250 + 200	25.4	*Pichia fermentans*
VI	500	150 + 100 + 80 + 80	380 + 120	200 + 190 + 110	1.7	*Saturnispora* sp.
VII	530	280 + 250	450 + 80	250 + 250 + 30	1.3	*Cryptococcus carnescens*
VIII	550	280 + 190 + 80	550	300 + 180 + 70	0.4	*Cryptococcus flavescens*
IX	600	275 + 185 + 80	590	295 + 295	0.9	*Zygotorulaspora florentinus*
X	600	250 + 240 + 110	520 + 80	250 + 190 + 170	1.3	*Cystofilobasidium infirmominiatum*
XI	625	300 + 265 + 60	400 + 115 + 90	320 + 300	2.5	*Meyerozyma guilliermondii*
XII	630	590	630	320 + 320	0.9	*Candida glaebosa*
XIII	630	295 + 295	420 + 140 + 80	320 + 320	0.4	*Candida oleophila*
XIV	640	320 + 240 + 80	425 + 215	340 + 225 + 75	1.3	*Rhodotorula mucilaginosa*
XV	730	315 + 315	500 + 230	250 + 200 + 150 + 100+30	0.4	*Hanseniaspora* sp.
XVI	775	320 + 310 + 105	775	385 + 200 + 160 + 80	5.5	*Hanseniaspora uvarum*
XVII	775	290 + 160 + 140 + 100 + 70	480 + 130 + 105 + 90	385 + 385	0.4	*Hanseniaspora osmophila*
XVIII	775	670 + 105	775	250 + 220 + 160 + 110	0.9	*Hanseniaspora valbeyensis*

[a] Taxonomic name obtained through the sequence of the 26S rRNA gene' D1/D2 region of a representative number of isolates of each group.

neoformans, were found in any sample analyzed. However, yeasts have been related with the development of some allergies, inflammatory bowel disease and infections in immunologically affected individuals (Loureiro and Querol, 1999). On the other hand, some of the isolated yeasts have been associated to biological control in different situations.

The microbial populations found in this study (AM, PM, TC, LAB and molds and yeasts) tended to be higher than other studies. There are certainly differences in the type and characteristics of the samples (whole fruits, cut fruits, degree of maturation, cultivar, among others), the points of the food chain where the samples were collected and the extension of the production and distribution chains and marketing period. The microbial quality of fresh-cut fruits may also reflect the great diversity of conditions prevailing during cultivation, harvesting and processing. Nevertheless, regarding the apple samples analyzed during the present study, none of the samples analyzed prior to their best-before date were spoiled, based on visual appearance. On the contrary, the apples slices studied after the expiration date showed some signs of visual spoilage (brownish color, texture, presence of gas, dehydration) and a significant increase (t test, $p < 0.002$) in the levels of yeasts was detected.

Most microorganisms have difficulty penetrating the skin of intact fruits but when protective barriers are destroyed they can colonize tissues and grow resulting in a spoilage problem or a public health threat. Another important aspect related with the microbial quality of fresh-cut fruits is the maintenance of refrigeration temperatures. This may lead us to think that during the production chain, temperature abuses may arise allowing conditions for certain groups of microorganisms to grow. Abuses of temperature during any phase of the processing, distribution or selling will increase microbial load especially psicrotrophic microorganisms and yeasts as some of them have a psychrotrophic profile.

4. Conclusion

The present study is the first work carried out to provide information on microbial quality of fresh-cut apple available in Portugal. The results obtained revealed high levels of the different groups of microorganisms, though none of the samples were positive for *E. coli*, *C. sakazakii*, *Salmonella* spp. and *L. monocytogenes*. According to the regulation of the European Commission (EC, 2005, EC, 2007) the apple samples fulfilled the microbiological criteria of food hygiene (manufacturing process) and safety (market place). The presence of coliforms (100%) and staphylococci (5.8%) in the samples indicates the potential of this kind of food becoming a microbial vehicle, as well as highlighting the need to improve control measures and hygiene in the selling, distribution and production of fresh-cut apple. A great diversity of yeast species was identified and some of them associated with food spoilage. However, opportunistic yeast pathogens were not detected. Fresh-cut fruit processors should be aware that the adherence to good manufacturing practices programs will result in fruit with lower microbial populations of mesophilic and psychrotrophic bacteria and yeasts, thus increasing overall quality. It is important to reduce the risk of spoilage, which is often associated with a low organoleptic profile, short shelf-life, high economic losses as well as increased public health risks.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fm.2015.06.003.

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Capítulo III

Microbiological quality and safety of minimally processed fruits in the marketplace of Southern Portugal

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Microbiological quality and safety of minimally processed fruits in the marketplace of southern Portugal

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ABSTRACT

The availability of fresh-cut fruit (FCF) in the marketplace has been increasing in Portugal, although reports of its microbial quality are not known. Due to the growing concerns of these commodities over their microbial safety, the objectives of this work were to study the microbiological quality and prevalence of *Salmonella* and *Listeria monocytogenes* on fresh-cut fruits sold in southern Portugal. A study to examine the changes in pH and microbial counts, before and after the expiration dates, was also made. A total of 160 samples was purchased in the local grocery stores between September 2011 and August 2014, before their sell-by date. These samples were assayed for aerobic mesophilic (AM) and psychrotrophic (AP) microorganisms, yeasts and molds (YM), lactic-acid bacteria (LAB), coliforms (TC), *Escherichia coli* and coagulase positive staphylococci as well as *L. monocytogenes* and *Salmonella*. The microbiological counts ranged from 3.0-9.2 lg cfu/g (AM); 2.2–10.7 lg cfu/g (AP); 2.3–10.4 lg cfu/g (YM); 1.9–9.0 lg cfu/g (LAB) and less than 1–9.1 lg cfu/g (TC). The melons and watermelon presented the highest levels of the microbial quality parameters studied. However, no *E. coli*, staphylococci, *Salmonella and L. monocytogenes* were detected in any of the samples. After the sell-by date, an increase of the AM, AP, LAB and YM values was observed in all fruits. Conversely, the differences found in TC counts before and after the best-before date had no statistical significance. A decrease in pH was observed in all fruits except pineapple whose pH slightly increased after 14 days of storage. The results highlight the importance of preventing contamination and cross contamination, selecting adequate decontamination technologies and maintaining a strict temperature control during processing, distribution and selling of FCF.

1. Introduction

During the last few decades, fresh produce, including fruits, have emerged as a new vehicle for the transmission of food-borne diseases associated with etiological agents that in the past were ascribed to animal reservoirs. An increase in outbreaks has been reported all over the world (Denis, Zhang, Leroux, Trudel, & Bietlot, 2016; Sivapalasingam, Friedman, Cohen, & Tauxe, 2004). Some of the last outbreaks attributed to *Salmonella enterica* in fresh fruit include *Salmonella* Braenderup in mangoes (CDC, 2012a), *Salmonella* Typhimurium and *Salmonella* Newport in cantaloupe (CDC, 2012b), *Salmonella* Agona in papayas (CDC, 2011a) and *Salmonella* Newport in watermelon ready to eat (Byrne et al., 2014). Pathogenic *Escherichia coli* has also been found in outbreaks involving fresh produce through the consumption of raw sprouts (CDC, 2014), ready-to-eat salads (CDC, 2013), organic spinach (CDC, 2012c) and romaine lettuce (CDC, 2010; 2012d). Additionally, listeriosis is among the most important cases of outbreaks in the United States involving the consumption of cantaloupe (CDC, 2011b). The factors that may have contributed to the emergence of fresh produce as a cause of outbreaks are the natural evolution of microorganisms, the changes in the industry, in the population characteristics and in consumer behavior. On the other hand, the enhanced epidemiological surveillance and the application of new techniques to identify and track the pathogens have increased, thus resulting in a greater awareness of food borne infections worldwide (Beuchat, 2002; Brandl, 2006; Tauxe et al., 1997). The emergence of

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pathogens with low infection doses and the ability of microorganisms acquiring virulence or pathogenicity factors through natural evolution mechanisms are fundamental aspects in understanding the rise of fresh food products as responsible for diseases associated in the past to animals' reservoirs (Croxen et al., 2013; Melo, Andrew, & Faleiro, 2015; van Elsas, Semenov, Costa, & Trevors, 2011). Within the industry, mass production and centralization of the production/processing systems generates large distribution networks implying an increase in volumes of importation/exportation of fresh fruits and vegetables and the rising of the fresh-cut industry, which may contribute to these problems (Denis et al., 2016). The increase in the at-risk populations (elderly, immune-compromised), and the consumption of minimally processed fruits and vegetables may also have led to increased outbreaks (Beuchat, 2002; Brandl, 2006; Denis et al., 2016; Tauxe et al., 1997).

The minimally processed fruits are characterized by having non-sterile cut surfaces which are physiologically active and rich in nutrients and water. These foods are raw, ready-to-eat and not subjected to any thermal or chemical process of preservation (Berger et al., 2010; Nguyen-the & Carlin, 1994; Olaimat & Holley, 2012). For this reason, fresh-cut fruits are susceptible to microbiological contamination in the various stages, from food processing to distribution and commercialization (Barth, Hankinson, Zhuang, & Breidt, 2009; Francis et al., 2012). If the processing operations are improper and storage and distribution occur in inadequate conditions (temperature), some microorganisms from the initial population or resulting from cross contamination, can survive and multiply, accelerating the degradation processes (in the case of spoilage microorganisms) and/or increasing the risk of food becoming a danger to public health (in the case of pathogenic microorganisms). Additionally, the trend to increase the shelf-life of refrigerated foods can allow the growth of psychrotrophic pathogens such as *Listeria monocytogenes* (Melo et al., 2015). This trend and the use of packaging techniques, such as modified atmosphere, contributes to inhibit the growth of aerobic microbiota but does not inhibit facultative anaerobic and anaerobic pathogenic microorganisms that may have the ability to survive and multiply under these conditions. The microbial growth on food products during the chain of production and commercialization are believed to be one of the main causes of the majority of outbreaks (Codex Alimentarius Commission, 1999) and spoilage. Furthermore, the ability of microbiota to subsist and/or grow at different temperatures in fresh-cut fruits have been described by several researchers (Abadias, Usall, Anguera, Solsona, & Viñas, 2012; Alegre, Abadias, Anguera, Oliveira, & Viñas, 2010; Alegre, Abadias, Anguera, Usall, & Viñas, 2010; Dingman, 2000; Salazar, Lourenço, Graça, Quintas, & Nunes, 2015; Santo, Graça, Nunes, & Quintas, 2016). The microbial quality of fresh-cut fruit is of concern, not only from the food safety point of view, but also due to the spoilage involved in the reduction of shelf-life which results in huge economic losses (Johnston et al., 2006). The presence of spoilage microorganisms, mainly yeasts, lactic-acid bacteria and pectinolytic pseudomonas, may explain the off-flavor formation, slimy surface, wetness, soft rot, changing color and visual microbial growth/colonies (Nguyen-the & Carlin, 1994). Those spoilage microorganisms may have a mesophilic or a psychrophilic behavior such as lactic-acid bacteria (Pothakos et al., 2014).

The main objective of the present work was to study the microbiological quality of fresh-cut fruits in the marketplace in southern Portugal (Algarve), through the enumeration of aerobic mesophilic and psychrotrophic microbiota, total coliforms and *E. coli*, lactic-acid bacteria, coagulase-positive staphylococci and fungi. The detection of the food safety parameters *Salmonella* spp. and *L. monocytogenes* was also an objective of this work. In addition, a characterization of the microbiological parameters (aerobic mesophilic and psychrotrophic microbiota, total coliforms, lactic-acid bacteria and fungi) of the fresh-cut fruits before and after their sell-by date was performed.

2. Material and methods

A total of 160 packed samples of fresh-cut fruits were bought in retail markets in Algarve (Portugal) before their sell-by date during the period of 2011 and 2014. Samples of individual packs (70 g - 100 g) of fruit pieces were purchased throughout the year and transported to the laboratory in refrigeration conditions. For each sample, date, place of manufacture and purchase, lot number and best-before date were registered. One hundred and five fresh-cut fruit samples were examined on the day of buying before their best-before date (pineapple: 29; mango: 9; papaya: 10; green melon: 19; cantaloupe melon: 7; Galia melon: 12; watermelon: 8; strawberries: 4; fruit salads: 7) for mesophilic (AM) and psychrotrophic (AP) aerobic counts, yeasts and molds (YM), lactic-acid bacteria (LAB), total coliforms (TC), *E. coli*, coagulase positive staphylococci (STAPH) and for the safety parameters *Listeria monocytogenes* and *Salmonella* sp.. Fifty-five fresh-cut samples (pineapple: 10; mango: 9; papaya: 6; green melon: 9; cantaloupe melon: 7; Galia melon: 9; watermelon: 5) were analyzed before (5–6 days) and after their best-before date (when visible spoilage was detected). The microbiological parameters studied were AM and AP, TC, LAB and YM. The pH values of the fruits were also studied in the fresh-cut fruit before and after the expiration date. The set of 55 packed samples of fresh-cut fruit studied after their expiration date were stored, unopened, at 4 °C for 10–14 days when observable spoilage was noticed. Then the packages of these samples were opened and analyzed for the microbial parameters. The pH values measurements were performed, before and after the expiration dates, using a digital Crison instrument, GLP 21 pH meter, equipped with a penetration electrode, at 21 °C. For each sample, the pH was measured in 2 pieces of fruit in triplicate.

2.1. Microbiological analysis

The microbiological quality of fresh-cut fruit samples was studied according to standard methodologies (ISO) summarized in Supplemental Table S1 and using Chromocult Agar (Merck, Darmstadt, Germany) for coliform and *Escherichia coli* (González, Tamagnini, Olmos, & Sousa, 2003).

Subsamples of twenty-five grams of each fruit package were diluted in 225 mL of buffered peptone water (BPW, Oxoid) and homogenized in a stomacher (Model 400 Circulator, Seward, Norfolk, England) for 2 min, at regular speed. The enumeration of AM, AP, LAB, TC and *E. coli*, YM and STAPH was performed from this suspension. The remaining suspension was incubated at 37 °C for 18 h for the detection of *Salmonella* sp.. The accounting of total coliform and *E. coli* was performed by pouring plating aliquots of the serial dilutions in Chromocult agar. Another portion of 25 g of the sample was homogenized in 225 mL Half-Fraser broth (Oxoid, Basingstoke, England) and incubated at 30 °C during 24 h for the detection of *L. monocytogenes*.

2.2. Pathogen confirmation

Presumptive isolates of *Salmonella* spp. and *L. monocytogenes* were collected from 5 to 2 samples of fresh-cut fruits, respectively. Colonies were streaked onto TSA (Tryptic Soy agar, Scharlau, Barcelona, Spain) until their purification and were then tested for Gram stain, cytochrome C oxidase and catalase activities. Presumptive colonies of *Salmonella* spp. were cultivated in Brain Heart

Infusion (Scharlau) and *L. monocytogenes* in TSB (Biokar Diagnostics, Beauvais, France) supplemented with yeast extract (0.6%) for further DNA extraction. The guanidine thiocyanate method (adapted from Pitcher, Saunders, & Owen, 1989) was used for DNA extraction of *Listeria* sp. isolates and the boiling method (Sambrook, Fritsch, & Maniatis, 1989) was employed for DNA extraction of *Salmonella* sp., presumptive isolates. PCR reactions with specific genus and species primers were achieved for the confirmation of the identity of the foodborne pathogens. The PCR mixtures (70 µl) were prepared [50–500 ng DNA, 0.5 µM each primer, 10 µM deoxynucleotides (Promega, Madison, USA), 1.5 mM $MgCl_2$ and 1 unit DNA polymerase (Promega)] and amplifications occurred in a thermocycler (My Cycler thermal cycler, BioRad, Hempstead, United Kingdom). Amplification products were analyzed in a 1% agarose gel stained with ethidium bromide with 1 × TAE buffer and observed under UV light in a G-Box Syngene-Genesis 10 UV Scanner (UK). Amplicons sizes were estimated by comparison against a DNA ladder (NZYDNA Ladder 5, NZYTech, Lisbon, Portugal).

2.2.1. *Salmonella spp.*

Salmonella spp. presumptive colonies were studied through the amplification of the gene *invA* encoding proteins of the type III secretion system which has been associated to the ability of *Salmonella* to invade epithelial cells. The primers 139f 5′-GTGAAATTATCGCCACGTTCGGGCAA-3′ and 141r 5′-TCATCGCACCGTCAAAGGAACC-3′ were selected to perform the PCR reactions and the PCR products generated has 285 bp (Rahn et al., 1992). The amplification started with the denaturation step at 95 °C for 1 min, followed by 38 cycles of denaturation at 95 °C for 30 s, annealing at 64 °C for 30 s, extension at 72 °C for 30 s and a final extension at 72 °C for 4 min (Malorny et al., 2003).

2.2.2. *L. monocytogenes*

L. monocytogenes presumptive colonies were studied with two primers' pairs resultant from the *iap* gene that encodes for invasion-associate protein (protein p60). The first pair of primers were specific for all *Listeria* species: UnilisA (5′-GCTACAGCTGGGATTGCGGT-3′) and Lis1B (5′-TTATACGCGACCGAAGCCAA-3′). The second primers' pair was specific for the species *L. monocytogenes*: MonoA (5′-CAAACTGCTAACACAGCACT-3′) and MonoB (5′-GCACTTGAATTGCTGTTATTG-3′). The amplification began with an initial denaturation step for 3 min at 94 °C, followed by 30 cycles of denaturation for 1 min at 94 °C, annealing for 45 s at 56 °C and an extension at 72 °C during 45 s. A final elongation period at 72 °C, occurred for 5 min. The *Listeria* genus PCR product has 1.4 kb and the *L. monocytogenes* PCR product has about 400 bp (Bubert et al., 1997).

2.3. *Data analysis*

Results of microorganism's counts (lg cfu/g) and pH measurements are presented as the mean ± standard error. Microorganisms mean abundances (in lg cfu/g) and pH values of samples prior to best-before date were compared to packages sampled after their expiration date using the t-Student test for two independent samples. The two-sample *t*-test is very robust to normality and to constant variances of sample data while accounting for the amount of available information via the degrees of freedom (Vining & Kowalski, 2011).

3. Results and discussion

3.1. *Microbiological analysis*

The microbial quality of fresh-cut fruit was performed on samples purchased at the marketplace in southern Portugal (Algarve) in the largest national supermarkets. The aerobic mesophilic (AM) mean counts of the studied fruits ranged from 3.0 to 9.2 lg cfu/g (Table 1). Only 3.0% of the samples had counts higher than 8 lg cfu/g and 52.0% lower than 5.0 lg cfu/g. Watermelon, mango and cantaloupe melon were the fruits with the highest AM mean counts (6.4, 5.6 and 5.5 lg cfu/g, respectively). The lowest AM counts were found on fresh-cut pineapple, with 81.5% of samples having AM counts lower than 5 lg cfu/g. Graça et al. (2015) described results on fresh-cut apples where AM counts ranged from 3.3 to 8.9 lg cfu/g. However, only 2.9% of the apple samples had AM counts inferior to 10^5 cfu/g and 59.7% of samples had counts between 10^7 and 10^8 cfu/g. Abadias, Usall, Anguera, Solsona, and Viñas (2008) found lower values in fresh-cut fruit samples studied in Catalonia (Spain) with results that varied between 2.0 and 7.1 lg cfu/g and 90.4% of their samples had AM counts inferior to 5 lg cfu/g. On the contrary, a study conducted by Viswanathan and Kaur (2001) found higher counts of AM on pineapple (6.9–8.2 lg cfu/g) and watermelon (6.9 and 8.0 lg cfu/g) when compared to those obtained in the present work.

The enumeration of aerobic psychrotrophic microorganisms (AP) is presented in Table 2. The AP mean counts ranged from 2.2 to 10.7 lg cfu/g and 37.1% of the samples had counts lower than 5 lg cfu/g. Cantaloupe melon, green melon and Galia melon had the highest mean AP counts (6.2, 5.7 and 5.6 lg cfu/g, respectively). AP counts obtained were higher than the ones obtained by Graça et al. (2015) on fresh-cut apples and by Abadias et al. (2008) on fresh-cut fruits, where the highest counts were 8.4 and 7.1 lg cfu/g, respectively. Whether the number of psychrotrophic microorganisms is high or low it should be emphasized that these microbes are able to grow at refrigeration temperatures, thus contributing to the spoilage of the fruits. However, not all psychrotrophic species cause food spoilage. In addition, if this group contains any pathogen species they will also be able to grow.

The total plate counts (AM and AP) are useful indicators of existing favorable conditions of multiplication of microorganisms, of efficient application of good hygienic practices and adequate temperature control during the entire food production chain (Aycicek, Oguz, & Karci, 2006).

YM counts (Table 3) were lower than the levels of mesophilic and psychrotrophic populations. The range in which this microbial group was found in the fresh-cut fruit studied was 2.3–10.4 lg cfu/g.

Only 2.8% of the fresh-cut fruit samples had counts superior to 8 lg cfu/g and 62.9% of the samples had counts inferior to 5 lg cfu/g. Strawberries, pineapples and mango, presented the highest mean YM counts (5.2, 5.1 and 4.7 lg cfu/g, respectively). Tournas, Heeres, and Burgess (2006) observed higher YM contamination levels on fresh-cut cantaloupe, pineapple, watermelon and mixed salads with counts higher than 6.0 lg cfu/g and lower in fresh-cut strawberries (4.4 lg cfu/g). Graça et al. (2015) also found high YM counts on fresh-cut apple samples with values ranging from 3.6 to 7.1 lg cfu/g. On the contrary, fresh-cut fruit samples analyzed by Abadias et al. (2008) had lower levels of YM contamination with counts between 1.7 and 4.9 lg cfu/g. As in the case of the study conducted by Graça et al. (2015) on fresh-cut apples, this microbial population consisted mainly of yeasts. According to Tournas et al. (2006) the presence of high levels of yeasts may result from high contaminated raw fruits or lack of hygienic practices.

Regarding LAB counts (Table 4), a great percentage of the samples had a contamination inferior to 5 lg cfu/g (67.6%) and only 1.0% had counts superior to 8 lg cfu/g. The highest means of LAB were found in watermelon, papaya and green melon (5.9, 5.2 and 5.1 lg cfu/g, respectively). Graça et al. (2015) observed higher contamination levels of LAB on fresh-cut apples with counts between 2.8 and 8.7 lg cfu/g. In the case of the fresh-cut fruit analyzed by Abadias et al. (2008) the results observed were much lower with

Table 1
Aerobic mesophilic microorganisms (AM) in the fresh-cut fruit samples analyzed.

	Percentage of samples in the indicated interval							
	n	$<10^5$ [a]	$10^5–10^6$	$10^6–10^7$	$10^7–10^8$	$\geq 10^8$	Range[b]	Mean[b]
Total	100	52.0	26.0	14.0	5.0	3.0	3.0–9.2	5.2
Pineapple	27	81.5	14.8	0.0	3.7	0.0	3.0–7.2	4.5
Mango	9	66.7	0.0	11.1	0.0	22.2	3.9–9.2	5.6
Papaya	9	55.6	33.3	0.0	11.1	0.0	4.0–7.4	5.1
Green melon	17	52.9	23.5	11.8	5.9	5.9	3.7–8.9	5.3
Galia melon	12	41.7	25.0	33.3	0.0	0.0	4.6–6.8	5.3
Cantaloupe melon	7	14.3	71.4	14.3	0.0	0.0	4.5–6.6	5.5
Watermelon	8	0.0	37.5	37.5	25.0	0.0	5.2–7.5	6.4
Strawberry	4	50.0	25.0	25.0	0.0	0.0	3.2–6.9	5.0
Fruit salads	7	28.6	42.9	28.6	0	0	4.1–6.8	5.4

[a] Range in cfu per gram of fruit.
[b] Counts are given in terms of lg cfu/g.

Table 2
Aerobic psychrotrophic microorganisms (AP) in the fresh-cut fruits analyzed.

	Percentage of samples in the indicated interval							
	n	$<10^5$ [a]	$10^5–10^6$	$10^6–10^7$	$10^7–10^8$	$\geq 10^8$	Range[b]	Mean[b]
Total	105	37.1	32.4	17.1	9.5	3.8	2.2–10.7	5.5
Pineapple	29	36.7	36.7	10.0	10.0	3.3	2.5–8.7	5.4
Mango	9	44.4	33.3	11.1	11.1	0.0	4.4–7.0	5.4
Papaya	10	50.0	30.0	0.0	20.0	0.0	3.3–8.0	5.2
Green melon	19	36.8	15.8	31.6	10.5	5.3	2.7–8.8	5.7
Galia melon	12	41.7	25.0	25.0	0.0	8.3	3.9–10.7	5.6
Cantaloupe melon	7	14.3	42.9	14.3	14.3	14.3	3.3–10.6	6.2
Watermelon	8	25.0	5.0	25.0	0.0	0.0	2.3–6.5	5.3
Strawberry	4	25.0	50.0	25.0	0.0	0.0	4.0–6.9	5.4
Fruit salads	7	42.9	28.6	14.3	14.3	0.0	2.2–7.3	4.9

[a] Range in cfu per gram of fruit.
[b] Counts are given in terms of lg cfu/g.

Table 3
Yeasts and molds (YM) in the fresh-cut fruits analyzed.

	Percentage of samples in the indicated interval							
	n	$<10^5$ [a]	$10^5–10^6$	$10^6–10^7$	$10^7–10^8$	$\geq 10^8$	Range[b]	Mean[b]
Total	105	62.9	25.7	4.8	3.8	2.8	2.3–10.4	4.7
Pineapple	29	44.8	41.4	6.9	6.9	0.0	2.6–7.3	5.1
Mango	9	77.8	11.1	0.0	0.0	11.1	3.5–8.1	4.7
Papaya	10	70.0	10.0	0.0	20.0	0.0	2.7–7.5	4.6
Green melon	19	78.9	15.8	5.3	0.0	0.0	2.3–6.6	4.1
Galia melon	12	75.0	16.7	0.0	0.0	8.3	3.1–10.0	4.5
Cantaloupe melon	7	85.7	0.0	0.0	0.0	14.3	2.7–10.4	4.6
Watermelon	8	50.0	50.0	0.0	0.0	0.0	3.2–5.6	4.6
Strawberry	4	50.0	25.0	25.0	0.0	0.0	3.8–6.6	5.2
Fruit salads	7	42.9	42.9	14.3	0.0	0.0	2.5–6.7	4.6

[a] Range in cfu per gram of fruit.
[b] Counts are given in terms of lg cfu/g.

counts between 1.7 and 4.8 lg cfu/g.

TC values (Table 5) ranged from less than 1.0 to 9.1 lg cfu/g and 77.2% of the samples analyzed had counts lower than 5 lg cfu/g. The highest TC mean values were found on watermelon, cantaloupe and papaya (5.2, 4.8 and 4.4 lg cfu/g). Viswanathan and Kaur (2001) conducted a study where they obtained counts of TC from 4.3 to 7.9 lg cfu/g in pineapples and between 4.5 and 6.9 lg cfu/g in watermelon samples. In the study by Graça et al. (2015) TC were counted in fresh-cut apples with values ranging from 1.8 to 7.6 lg cfu/g. Coliforms occur naturally on produce and thus their importance as fecal indicators is limited, as some species may be of non-fecal origin (Doyle & Erickson, 2006). Additionally, none of the samples studied contained *E. coli*, therefore, the fruit samples analyzed complied with the microbiological criteria of food hygiene according to the regulation of the European Commission (EC, 2005; EC, 2007).

Regarding the food safety parameters at the marketplace (EC, 2005; EC, 2007), *L. monocytogenes* and *Salmonella* spp. were not detected in the fresh-cut fruit samples studied, which is consistent with other published studies where pathogens were in very low numbers or non-existent (Abadias et al., 2008; Denis et al., 2016; Graça et al., 2015; Johnston et al., 2006; Santos et al., 2012). Additionally, the coagulase positive staphylococci were also not detected. This desirable situation, regarding the absence of those pathogenic microorganisms, clearly depends on the appropriate processing conditions (respecting good manufacturing practices)

Table 4
Lactic-acid bacteria (LAB) in the fresh-cut fruits analyzed.

	Percentage of samples in the indicated interval							
	n	$<10^5$ [a]	10^5-10^6	10^6-10^7	10^7-10^8	$\geq 10^8$	Range[b]	Mean[b]
Total	102	67.6	17.6	9.8	3.9	1.0	1.9–9.0	4.5
Pineapple	29	82.8	13.8	3.4	0.0	0.0	2.0–6.8	3.8
Mango	9	88.9	0.0	0.0	0.0	11.1	1.9–9.0	4.4
Papaya	10	40.0	40.0	10.0	10.0	0.0	3.3–7.7	5.2
Green melon	16	62.5	6.3	25.0	6.3	0.0	3.4–7.6	5.1
Galia melon	12	75.0	16.7	8.3	0.0	0.0	2.9–5.8	4.4
Cantaloupe melon	7	71.4	28.6	0.0	0.0	0.0	3.4–5.8	4.3
Watermelon	8	25.0	37.5	12.5	25.0	0.0	4.8–7.3	5.9
Strawberry	4	75.0	0.0	25.0	0.0	0.0	2.5–6.7	4.3
Fruit salads	7	57.1	28.6	14.3	0.0	0.0	3.6–6.6	4.9

[a] Range in cfu per gram of fruit.
[b] Counts are given in terms of lg cfu/g.

Table 5
Total coliforms (TC) in the fresh-cut fruits analyzed.

	Percentage of samples in the indicated interval							
	n	$<10^5$ [a]	10^5-10^6	10^6-10^7	10^7-10^8	$\geq 10^8$	Range[b]	Mean[b]
Total	101	77.2	12.9	5.9	2.0	3.0	ND-9.1	3.7
Pineapple	27	88.9	3.7	3.7	0.0	3.7	ND-9.1	2.4
Mango	9	77.8	0.0	0.0	0.0	22.2	0.7–8.4	4.3
Papaya	9	77.8	0.0	11.1	11.1	0.0	1.0–7.7	4.4
Green melon	18	72.2	16.7	11.1	0.0	0.0	0.7–6.6	4.1
Galia melon	12	75.0	25.0	0.0	0.0	0.0	1.7–5.5	4.2
Cantaloupe melon	7	42.9	57.1	0.0	0.0	0.0	3.4–6.0	4.8
Watermelon	8	62.5	12.5	12.5	12.5	0.0	4.1–7.0	5.2
Strawberry	4	100.0	0.0	0.0	0.0	0.0	3.2–4.5	3.7
Fruit salads	7	71.4	14.3	14.3	0.0	0.0	ND-6.5	4.1

ND-less than 1.0 lg cfu/g.
[a] Range in cfu per gram of fruit.
[b] Counts are given in terms of lg cfu/g.

and the storage under strict refrigeration temperatures. Any abuse of the temperatures may permit the growing of eventual foodborne bacteria to levels responsible for diseases, especially in case storage occurs during long periods of time, which may allow psychrotrophic microorganisms, such as *L. monocytogenes*, to grow. However, foodborne bacteria may be inhibited by antimicrobial compounds of plant/fruit or microbial origin (Gyawali & Ibrahim, 2014). In reality, natural microbiota occurring on food has been recognized as being able to inhibit foodborne bacteria due to the production of antimicrobial substances (primary or secondary metabolites) or competition (Alegre et al., 2013).

The microbiological parameters reported for the FCF tended to be high, although the samples studied did not show visible signs of spoilage. In fact, the level of microorganisms counted in the fruits studied were, in general, higher than the results presented by Abadias et al. (2008) for fresh-cut fruits. However, these authors studied apple, peach, orange, mango and pineapple, whereas this present work also included papaya, different types of melons and watermelon, which are fruits with higher values of pH. These fruits had the highest levels of AM, AP, LAB and TC. Watermelon (pH-5.70) was the fruit that presented the highest counts of AM, LAB and TC and cantaloupe melon (pH-7.08) samples presented the highest values of AP and was the second most contaminated with TC. The melons, including watermelons and cantaloupe, have been linked to outbreaks and associated to high risk of microbial contamination and growth (Byrne et al., 2014). The microbial quality of fresh-cut fruit depends on the fruit variety, minimal processing operations and packaging and storage conditions. The survival and growth of microorganisms on the fruit are affected by various intrinsic and extrinsic factors. Among the intrinsic factors are the nutrient content, water activity (a_W), redox potential, texture of the tissues, the presence of antimicrobial compounds, as well as the pH and the buffering capacity. On the other hand, the survival and growth of microorganisms in fruit are also dependent on their physiological activity (viability, specific growth rate) and ability to adapt to stress conditions as well as their behavior in mixed populations (competition, antagonism and synergism) (Beuchat, 2002; Francis et al., 2012). However, the conditions dependent on processing operations may alter the previous factors contributing to changes in the microbial population. The operations of cutting, slicing, shredding, peeling increase the likelihood of contamination through cross contamination from other material/utensils/equipment during the various steps of production and processing. Additionally, the increased availability of sugars and other nutrients in minimally processed fruit contribute to changes in the microbiota and increase their population (Oms-Oliu et al., 2010; Pasha, Saeed, Sultan, Khan, & Rohi, 2014). The microbiota of raw material, the hygiene of the surfaces of materials and processing equipment, the manufacturing environment and the hygiene of food handlers, are major factors determining the microbiological quality and safety of the final product (Abadias et al., 2008; Johnston et al., 2005; Lehto, Kuisma, Maatta, Kymalainen, & Maki, 2011).

A study conducted in minimally processed vegetable plants showed high levels of total aerobic microorganisms on all surfaces of contact with food, especially in the peel equipment, knives and cutting boards (Lehto et al., 2011). The same authors have reported high counts of *Enterobacteriaceae* bacteria on the cutters and cutting boards. Although the production diagram may include washing and decontamination phases, it is difficult to obtain high reductions of the microbial loads (Beuchat, 1998). Johnston et al. (2006) reported increasing levels of *E. coli* from field samples to processing steps and even packaging in cantaloupe melon. The packaging and storage are also relevant steps that can provide conditions for contamination and growth of microorganisms in fruits and vegetables. Lehto et al. (2011) detected high values of total aerobic microorganisms in the atmosphere of the storage areas, processing and packaging.

3.2. Microbiological characterization of fresh-cut fruit before and after the best-before date

Fifty-five samples of fresh-cut fruit were studied before and after (10–14 days at refrigeration temperatures) their expiration

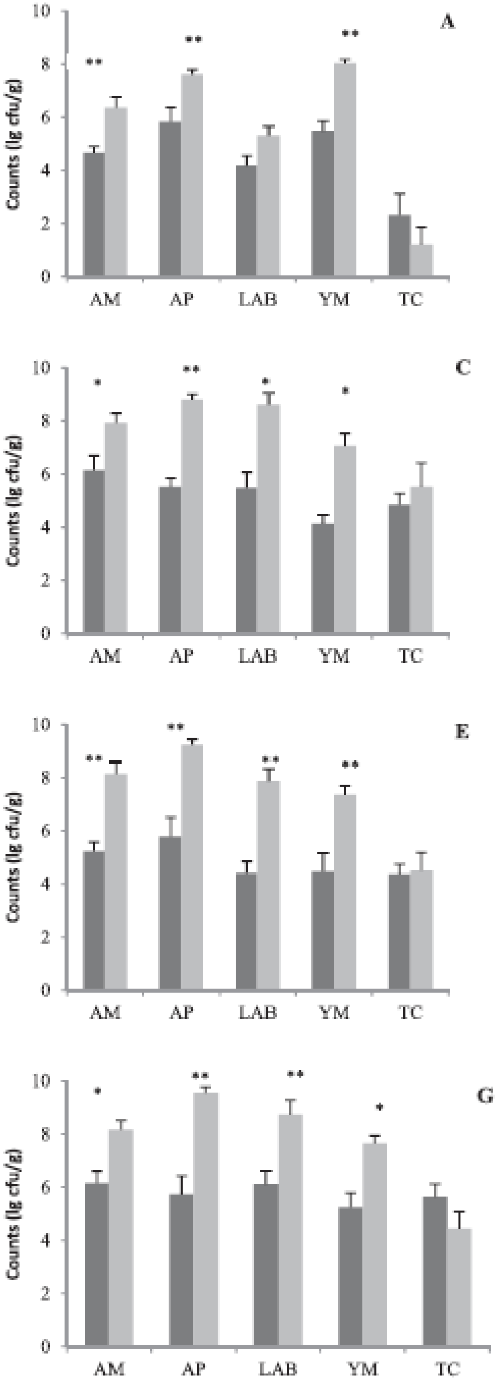

Fig. 1. Comparison of aerobic mesophilic (AM), psychrotrophic microorganisms (AP), lactic-acid bacteria LAB), yeasts and molds (YM) and total coliform counts (TC) (lg cfu/g, mean) in fresh-cut pineapple (A), mango (B), papaya (C), green melon (D), Galia melon (E), Cantaloupe melon (F) and watermelon (G), before (dark column) and after (white column) the best before date. t-Student test, $^{*}p < 0.05$ and $^{**}p < 0.005$. Vertical bars indicate standard error of the mean.

date, when spoilage characteristics were observed, for mesophilic and psychrotrophic aerobic counts, LAB, yeasts and molds and total coliforms. The results are shown in Fig. 1 for pineapple (A), mango (B), papaya (C), green melon (D), Galia melon (E), cantaloupe (F) and watermelon (G). Additionally, the pH was also measured in the same samples, before and after the expiration date (Fig. 2). After the expiration date, an increase of the AM, AP, LAB and YM values was observed in all the fruits studied and the differences registered were statistically significant (*t* test, $p < 0.05$), with the exception of LAB counts in pineapple. The number of psychrotrophs enumerated was high in all the FCF samples as expected for this group of microorganisms, which grows at the refrigeration temperatures recommended in the package label. Additionally, the number of mesophiles was also high indicating that some of this population is also able to grow at storage temperatures.

Regarding the determinations of TC after the expiration date, their values had slightly increased in papaya, green melon and Galia melon, slightly decreased in pineapple, cantaloupe melon and watermelon but unchanged in mango. However, the differences detected were not statistically significant (*t* test, $p > 0.05$) when comparing TC microbial counts before and after their best-before date. Graça et al. (2015) obtained similar results in fresh-cut apples, where there was a small increase in AM, AP, LAB and YM counts but not in TC counts after the expiration date. Competition and antagonism behaviors among the various groups of microorganisms may certainly explain these results regarding the TC populations. In fact, LAB are known for antagonistic activities as referred later on.

In regards to the measurements of pH, an intrinsic factor that strongly influences the survival and growth of microorganisms, the values obtained for the different fruits before the expiration date were as follows: 3.79 for pineapple, 4.41 for mango, 5.80 for papaya, 6.45 for green melon, 6.69 for Galia melon, 7.08 for cantaloupe melon and 5.70 for watermelon (Fig. 2). Pineapple and mango had pH values inferior to 5, however, papaya, green melon, Galia melon, cantaloupe melon and watermelon, had pH values higher than 5 and will more easily support the growth of microorganisms including pathogens. After the expiration date under refrigeration (Fig. 2), a decrease in pH values was observed in all the fruits with the exception of pineapple, whose pH value slightly increased even after 14 days. In the cases where a significant reduction of the pH was measured, a significant increase of the LAB growth was also registered. LAB are known to be present in the normal microbiota of fruits and have been associated to their spoilage, including the production of unpleasant odors (Fleet, 1992; Nguyen-the & Carlin, 1994; Pothakos et al., 2014). Their homolactic and heterolactic fermentative profiles will certainly contribute to the lowering of the pH due to lactic and acetic acid formation and development of off-flavors similar to buttermilk due to the production of acetylmethylcarbinol and 2,3 butanedione (diacetyl). The heterolatic metabolism of some LAB may be responsible for the production of ethanol, acetic acid, formic acid, diacetyl, 3 hydroxibutanone, 2,3 butanediol, acetaldehyde and CO_2, which are also involved in the degradation of fruits (Barth et al., 2009; Pothakos et al., 2014). LAB have been detected in several fresh-cut products, including honeydew, papaya, pineapple, cantaloupe, cabbage, carrots, chicory, celery, bell peppers and salad mixes (Allende, Jacxsens, Devlieghere, Debevere, & Artés, 2002; O'Connor-Shaw, Roberts, Ford, & Nottingham, 1994). Psychrotrophic LAB adapted to industrial environments can also get in contact with food products during their manufacturing process (Björkroth, 2005; Pothakos et al., 2014). According to Pothakos et al. (2014), LAB dominance at the end of shelf-life suggests fast growth during storage conditions even if initial populations are low. On the other hand, LAB have been used as biological control agents due to the fact they are able to limit the growth of accompanying microbiota and even pathogenic microorganisms (Gerez, Torres, Font de Valdez, & Rollán, 2013; Trias, Bañeras, Badosa, & Montesinos, 2008). Besides LAB, as referred by Francis et al. (2012) and Alegre et al. (2013), fresh-cut produce may harbor large populations of microorganisms that may possess antagonistic activity against other microorganisms including pathogens. In the case of pineapple and apple, the pH value measured after the best- before date, slightly increased from 3.79 to 3.88 and from 4.10 to 4.30, respectively, and the differences found in the number of LAB were not significant. In the fruits, especially in the ones with the lower pH, the other major microbial spoilage group found in the fresh-cut fruit samples was yeast. These microorganisms are able to grow in acidic foods and ferment sugars into alcohols, being responsible for off-flavors and off-odors (Barth et al., 2009; Tournas et al., 2006). Additionally, some of them may have also a psychrotrophic profile allowing then to increase their population at storage temperature (Graça et al., 2015). The combination of Good Agriculture and Manufacturing Practices with efficient decontamination technologies and the maintenance of a strict cold chain throughout the production, distribution and consumption are necessary to protect the safety, decrease the spoilage and improve the quality of minimally processed fruits.

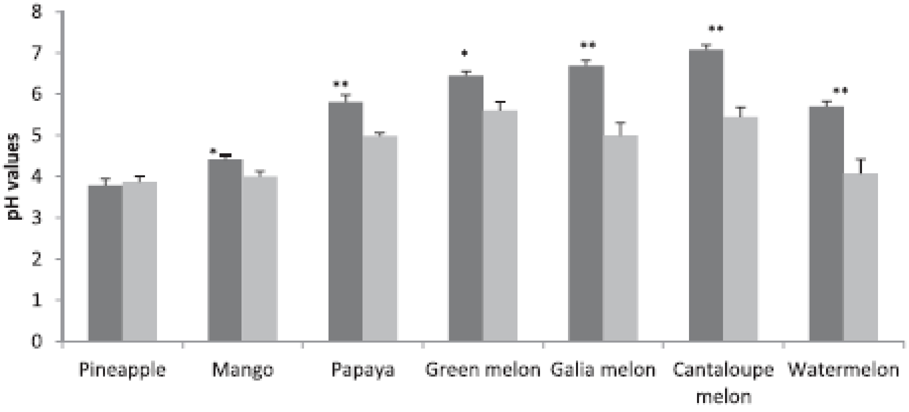

Fig. 2. Comparison of pH values in fresh-cut pineapple, mango, papaya, green melon, Galia melon, Cantaloupe melon and watermelon, before (dark column) and after (white column) the best before date. t-Student test, $^{*}p < 0.05$ and $^{**}p < 0.005$. Vertical bars indicate standard error of the mean.

4. Conclusions

The microbial quality parameters of the FCF studied in the marketplace in southern Portugal tended to be high, although the samples studied did not show visible signs of spoilage. Additionally, *E. coli*, coagulase positive staphylococci, *Salmonella* and *L. monocytogenes* were not detected, thus the fresh-cut samples studied fulfilled the European legislation according to the microbiological criteria of hygiene and safety (EC, 2005, 2007). The highest levels of AM, LAB and TC were found in watermelon, AP on cantaloupe and YM on strawberry, pineapple and mango. After the expiration date, in all the fruits, the values of AM, AP, LAB and YM increased, TC changed slightly, and the pH decreased with the exception of pineapple. Obtaining minimally processed microbiologically safe fruits implies the maintenance of good agricultural conditions as well as the adoption of good manufacturing practices at all pre-harvest, harvest and post-harvest stages. Good microbial quality of fresh-cut fruit is of concern, not only from the food safety point of view, but also due to the spoilage involved in the reduction of shelf-life that may result in enormous economic losses. Producers, processors, distributors, retailers and consumers must adopt good manufacturing/handling practices and good hygiene in order to prevent contamination, recontamination and microbial growth. All the phases should have a set of control measures, as producing microbiologically safe food is mandatory.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.foodcont.2016.09.046.

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Capítulo IV

Low dose UV-C illumination as an eco-innovative disinfection system on minimally processed apples

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Low dose UV-C illumination as an eco-innovative disinfection system on minimally processed apples

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ABSTRACT

In this study, the efficacy of UV-C illumination for inactivate *Escherichia coli*, *Listeria innocua* or *Salmonella enterica*, individually or in a mixture, *in vitro* and on apple slices was determined. Apple slices inoculated with a 10^7 cfu/mL suspension of above indicated pathogens were irradiated on both sides with UV-C illumination, with doses of 0.5 and 1.0 kJ/m^2. UV-C illumination disinfection efficacy was compared to that of washings with sodium hypochlorite at 100 ppm of free chlorine and with distilled water. Bactericidal activity of each treatment was assessed after 30 min and after 7 and 15 days of storage at 4 °C. Results showed that UV-C illumination at 1.0 kJ/m^2 could be an alternative to the wash with hypochlorite solutions. On the *in vitro* study, these doses completely inhibited the growth of the three bacteria either as pure cultures or in a mixture. In fresh-cut apple, the pathogens were also affected by the UV-C illumination, the 1.0 kJ/m^2 dosage being the one that resulted in higher bacteria inhibition in almost every case. The UV-C treatment did not affect the quality properties of fresh-cut apple.

1. Introduction

Consumption of fresh produce has increased over recent years, due to a tendency of adopting healthier food habits, and has led to the appearance of minimally processed (fresh-cut) products that are ready-to-eat. However, this growing demand raises the need for increase shelf-life and safety of these products. Fresh produce can be a vehicle for the transmission of foodborne pathogens, since they can be easily contaminated with microorganisms during production and processing. Contamination levels after harvesting can range from 3 to 7 log units, depending on the season and type of product (Ölmez and Kretzschmar, 2009).

In recent years, the number of outbreaks of human infections associated with the consumption of minimally processed products and unpasteurized fruit juices has increased. The major concerns are with enteric pathogens such as *Escherichia coli* O157:H7 and *Salmonella* spp. that have fast growth rates and low infectious doses (Martin, 2007). Salmonellosis and *E. coli* O157:H7 infection have been linked to watermelon, tomatoes, seed sprouts, melons, apple or orange juice (Blostein, 1993; del Rosario and Beuchat, 1995; Beuchat, 1996; Butler, 2000; Krause et al., 2001; Greene et al., 2008; Munnoch et al., 2009; Muranyi, 2012). Furthermore, listeriosis remains a great public health concern, as it has one of the highest case fatality rates of all the foodborne infections in Europe (20–30%) (Martin, 2007). The increase in reported outbreaks related to fresh fruit and vegetables may be the result of several causes. The per capita consumption of fresh produce has increased in developed countries. The demand for fresh produce year-round implies an increase in imports of these products from countries with different hygienic and sanitary conditions as well as the introduction of other pathogens (Lynch et al., 2009). Changes in processing, with more in-field cutting, coring and packaging, changes in the distribution systems, pathogens with different level of virulence, immunological changes among population segments and global trade, could be other reasons for the increase in outbreaks.

Fresh-cut fruit are more susceptible to foodborne pathogens because their natural barriers are removed. Several studies have demonstrated that *E. coli* O157:H7, *Salmonella* spp. and *Listeria monocytogenes* could survive and/or grow in a range of minimally processed fruit such as apples (Dingman, 2000; Alegre et al., 2010a), honeydew melon (Leverentz et al., 2001, 2003), peaches (Alegre et al., 2010b), melon and pineapple (Abadias et al., 2012) and oranges (Lourenço et al., 2012) at temperature of 10 °C or higher.

Washing fresh-cut products with sanitizing solutions is the only step in which a reduction of microbial contamination can be achieved (Allende et al., 2009; Ölmez and Kretzschmar, 2009). Chlorine (sodium hypochlorite solution, 50–200 mg/L for 1–2 min) is the most common sanitizer used in fresh-cut industry (Beuchat, 1998).

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However, chlorination is related to environmental and human health risks. Chemical reactions of chlorine with organic matter may create toxic compounds, such as trihalomethanes (Allende et al., 2009; Gil et al., 2009). Adding to this, the scarcity of water resources is another environmental problem that has to be considered. Among the different industries, the food industry ranks third in water consumption and wastewater discharge rates, after the chemical and refinery industries (Casani et al., 2005). Thus there is a need to reduce or replace the use of chlorine as a disinfectant, particularly in the fresh-cut industry.

Ultraviolet light illumination is a non-thermal disinfection technology that can be used in fresh-cut industry. Is easy to use, is lethal to most type of microorganisms (Bintsis et al., 2000), does not generate chemical residues (Guerreo-Beltrán and Barbosa-Cánovas, 2004) and is a dry cold process that can be effective at low cost (Bachmann, 1975). UV-C light has maximal microbiocidal effect at 254 nm. UV-C doses ranging from 0.5 to 20 $\mathrm{kJ/m^2}$ inhibit microbial growth by inducing the formation of pyrimidine dimers which distort the DNA helix and block microbial cell replication (Escalona et al., 2010). Cells become unable to repair their damaged DNA and die. The effectiveness of UV-C seems to be temperature independent (in the range from 5 to 37 °C), but depends on the illumination incidence, determined by the structure and topography of the surface of treated produce (Bintsis et al., 2000; Gardner and Shama, 2000), the fluence ($\mathrm{J\,m^{-2}}$) and the relative position of the source and the sample.

The purpose of this work was to evaluate the effect of different doses of UV-C illumination on reducing the populations of *E. coli* O157:H7, *Listeria innocua* and *Salmonella enterica* subsp. *enterica* inoculated individually and in a mixture in *in vitro* and in fresh-cut apples.

2. Materials and methods

2.1. Bacterial strains

Non-toxicogenic strains of *E. coli* O157:H7 NCTC 12900, *L. innocua* CECT-910 and *S. enterica* subsp. *enterica* Michigan ATCC BAA-709 were used in this study. *L. innocua* has been used as a model organism for *L. monocytogenes* (Francis and O'Beirne, 1997). The bacterial strains were maintained on Tryptone Soy Agar medium (TSA, Oxoid) at 4 ± 1 °C. Prior to the experiments each microorganism was sub-cultured for 24 ± 2 h at 37 ± 1 °C on TSA and then in 50 mL of Tryptone Soy Broth medium (TSB, Oxoid) and incubated at 150 rpm for 24 ± 2 h at 37 ± 1 °C. To recover cells, each bacterium was centrifuged at 8000 rpm for 15 min and the pellet was resuspended in 50 mL of saline peptone [(8.5 g/L NaCl (Panreac) and 1 g/L peptone (Panreac)]. Inoculums with the appropriate concentration were prepared by adjusting the suspension according to a standard curve with a spectrophotometer (Spectrophotometer UV-Vis, 175 Shimadzu-UV160, USA) measuring the transmittance at 420 nm. Concentrations applied were confirmed using the Miles and Misra surface colony count method (1938), drops of 20 µL of 10-fold dilutions were placed in triplicate onto the surface of the TSA medium. Drops were left to be fully absorbed before inverting and incubating the plates at 37 ± 1 °C for 24 ± 2 h. Colonies were counted and by pathogen the results were expressed as a reduction of cfu/mL when compared with the population in control plates.

2.2. UV-C illumination conditions

The UV-C equipment consisted in a cabinet of 100 cm × 100 cm × 50 cm with two sets of 6 unfiltered germicidal lamps (Philips, TUV 25W G25 T8 Longlife). One set was suspended on the top and the other one was placed on the bottom of the UV-C chamber. The fresh-cut apples were placed between the UV-C lamps over a net. In order to determine the UV-C illumination intensity of the lamps, a radiometer (UVX Radiometer, UVP Inc., USA) was placed at the same distance as the commodities (15 cm). The applied UV-C intensity was calculated as a mean of 20 readings in different places taken at each side of the net. Light intensity was kept constant, and the applied doses varied by modifying the exposure time. Prior to use the UV-C lamps were allowed to stabilize by turning on 10 min before treatment. A ventilation device was installed in the back of the box to avoid temperature increase because of UV-C illumination.

2.3. *In vitro antimicrobial activity of UV-C illumination against E. coli, L. innocua, S. enterica or a mixture of the three microorganisms*

A 10^8 cfu/mL pure culture suspension of *E. coli*, *L. innocua*, *S. enterica* or a cocktail of the three microorganisms was prepared and series of 10-fold dilution were made in SP. The concentrated and the diluted cultures were plated in Petri dishes (three drops of 10 µL) onto a specific medium for each pathogen: Sorbitol MacConkey Agar (Biokar) for *E. coli*, and Hektoen Agar (Biokar) for *S. enterica*, and Palcam Agar (Biokar) for *L. innocua*.

Three sets of dishes were made. One set was submitted to UV-C illumination immediately after plate inoculation and the other was treated 4 h after inoculation. The UV-C doses applied were 0.5 and 1.0 $\mathrm{kJ/m^2}$. Untreated but inoculated plates consisted in the third set and were used as control. Petri dishes were incubated at 37 ± 1 °C for 48 ± 2 h. Colonies were counted and the results expressed as the reduction of cfu/mL of each pathogen when compared to control plates. Control plates were counted only in diluted culture.

The experiment was conducted three times.

2.4. *Fresh-cut apple preparation*

'Golden Delicious' apples used in this study were purchased in a packinghouse and stored at 0.5 ± 0.5 °C and 90% RH before processing. Apples were washed, sanitized by immersion, rubbed in a sodium hypochlorite solution (Panreac) at 0.5% for 30 s, and let to dry. Apples were then aseptically cut using a cutting instrument, suitable for apples, in pieces of 10 g each, without core tissue, and with skin on.

2.5. *Antimicrobial activity of UV-C illumination on fresh-cut apples inoculated with pure culture of E. coli, L. innocua or S. enterica*

Apple pieces were submerged into a 500 mL of *E. coli*, *L. innocua* or *S. enterica* pure culture suspension at 10^7 cfu/mL for 3 min with 150 rpm orbital agitation. Inoculated samples were air-dried in a laminar flowhood for 30 min before receiving the treatment. For each inoculated microorganism, apple pieces were divided into 5 batches of 12 pieces each. Two of the batches were treated for 5 min at 150 rpm orbital agitation in flasks containing 500 mL of distilled water (DW) or sodium hypochlorite solution (SH) at 100 mg/L of free chlorine (pH 6.5), determined by using a free and total chlorine photometer (model HI9133, HANNA Instruments, Woonsocket, RI, USA). After SH treatment, apple pieces were drained and rinsed with cold distilled water for 3 min at 150 rpm orbital agitation. These two batches were left to dry in a laminar flowhood for 30 min.

The other batches were treated with UV-C illumination at doses of 0.5 and 1.0 $\mathrm{kJ/m^2}$ each. Untreated but inoculated pieces were used as control.

Apple pieces from each treatment were divided into 3 batches (4 pieces each) and packed in oriented polystyrene lidded trays

(125 mm × 103 mm × 45 mm, Nupik, Barcelona). The concentration of each pathogen on apple pieces was determined before (BT) and after (AT) the treatment and after 7 and 15 days at 4 ± 0.5 °C. At each sample time, for each disinfection treatment, 10 g of apple pieces were transferred into sterile Stomacher bags and mixed with 90 mL of sterile saline peptone and homogenized for 2 min in a Stomacher400 (Seward, London). Homogenates were serially diluted in saline peptone and 20 µL drops in triplicate were plated onto the surface of the TSA medium using the Miles and Misra method (1938) and incubated at 37 ± 1 °C for 24 ± 2 h. Colonies were counted and the results expressed as log cfu/g of apples. For each condition four replications were performed and the experiment was repeated three times.

2.6. *Antimicrobial activity of UV-C illumination on fresh-cut apples inoculated with a bacterial mixture*

E. coli, *L. innocua* and *S. enterica* were prepared as previously described and mixed in a cocktail at 10^7 cfu/mL of each bacterium. Concentration of each microorganism was confirmed using the Miles and Misra method (1938), plating 20 µL drops of appropriately diluted culture on Sorbitol MacConkey Agar for *E. coli*, and Hektoen Agar for *S. enterica*, and incubated at 37 ± 1 °C for 24 ± 2 h and on Palcam Agar for *L. innocua* incubated at 37 ± 1 °C for 48 ± 2 h. Samples of apple pieces were inoculated by dipping into a 500 mL containing the mixture of the three bacteria for 3 min with 150 rpm orbital agitation and left to dry. Afterwards, fruit were treated and stored as previously described. To determine the concentration of each bacterium before and after treatment and 7 and 15 days after cold storage, the same procedure previously described was used, except that TSA medium was replaced by selective medium for each bacterium, as described above. In preliminary experiments growth of the pathogens, that were illuminated or not with UV-C, using TSA *versus* selective media at 37 °C had no effect on the experimental outcome. For each condition four replications were performed and the experiment was conducted four times.

2.7. *Quality parameters of treated fresh-cut apple*

Non-inoculated apple pieces were treated and storage as previously described to determine the effect of the treatments in fruit quality. Four trays were used for each treatment at each sample time, and color, soluble solid content and titratable acidity were determined. Surface color of apple slices was measured with a CR-300 Minolta chromameter (Minolta, Inc., Tokyo, Japan), using CIE L^*, a^*, b^* coordinates. Four apple pieces were evaluated for each tray and the surface on both sides of the apple pieces was measured for each replicate. The L^* value is a useful indicator of darkening during storage, either resulting from oxidative browning reactions or from increasing pigment concentrations. The a^* value is a measure of greenness is highly correlated with color changes of apple flesh (Goupy et al., 1995), a more positive a^* value means progressive browning and a more positive b^* value indicates more yellowing. Sapers and Douglas (1987) also suggested that enzymatic browning at the cut surfaces of apples could be monitored by measuring changes in reflectance L^* and a^* values, and that b^* values seemed to be unrelated to the extent of browning. The percentage of soluble solid (°Brix) apples slices were measured at 20 °C in juice extracted by crushing the apple slices using a refractometer (Atago Co. Ltd. Tokyo, Japan). Titratable acidity was measured in 10 mL of apple juice dilute in 10 mL of distilled water and titrated with 0.1 N of NAOH to a pH value of 8.1. Results were calculated as g of malic acid per liter.

Table 1
Population (log cfu/mL) of pure cultures of *Escherichia coli* O157:H7, *Listeria innocua* or *Salmonella enterica* or their mixture by different exposure doses of UV-C.

Microorganism	UV-C dose (kJ/m^2)				
	0	0.5		1.0	
		Time after inoculation (h)			
		0	4	0	4
Pure culture					
E. coli	8.1 a	0.9 b	0.9 b	0.0 b	0.0 b
L. innocua	8.1 a	0.7 b	1.0 b	0.6 b	0.8 b
S. choleraesuis	8.1 a	0.0 b	0.6 b	0.0 b	0.0 b
Mixture of pure culture					
E. coli	7.8 a	0.0 b	0.0 b	0.0 b	0.0 b
L. innocua	7.8 a	0.0 b	0.8 b	0.0 b	0.0 b
S. choleraesuis	7.8 a	0.0 b	0.0 b	0.0 b	0.0 b

Within type of cultures, different letters indicate significant differences ($P < 0.05$) between treatments.

2.8. *Statistical analyses*

The reported values of reduction in bacteria on apple pieces were calculated by subtracting the population of inoculated but untreated apples from the population after treatment at the same storage conditions. Values represent the means of the different experiments, with 4 replicates per treatment per experiment. Data were subjected to analysis of variance and Duncan's multiple range tests using SPSS v.16.0 software (SPSS Inc., USA). Significant differences in reduction values were established by the least significant difference at the 0.05 level of significance.

3. Results

3.1. *In vitro antimicrobial activity of UV-C illumination against E. coli, L. innocua, S. enterica or a mixture of the three microorganisms*

The effect of *in vitro* antimicrobial activity of UV-C illumination on the three pathogens as pure culture or in a cocktail is presented in Table 1. Microbial development of all pathogens was significantly inhibited by the two doses of the UV-C treatments (0.5 and 1.0 kJ/m^2) either in plates illuminated immediately or 4 h after inoculation. In plates illuminated with the UV-C dose of 1.0 kJ/m^2, total inhibition of all pathogens was achieved, except *L. innocua* in pure culture. Although *L. innocua* was the less sensitive microorganism to this dose of UV-C, no significantly differences were observed among treatments and type of cultures. When plates were submitted to a UV-C dose of 0.5 kJ/m^2 no differences were observed among treatments and in pure culture the inhibition of pathogen population was higher than 7.1 log cfu/mL. When in a mixture, total inhibition of pathogens population was observed, except for *L. innocua* treated with UV-C immediately after inoculation, where there was inhibition of 7.0 log cfu/mL.

3.2. *Antimicrobial activity of UV-C illumination on fresh-cut apples inoculated with E. coli, L. innocua or S. enterica*

Fig. 1 represents the survival and growth of a single culture of *E. coli*, *L. innocua* or *S. enterica* on fresh-cut apple treated with UV-C illumination (0.5 and 1.0 kJ/m^2), SH and DW and on untreated (Ck) stored up 15 days at 4 ± 0.5 °C. The viable cells recovered before treatments on inoculated apple slices were 4.39, 4.96 and 5.11 log cfu/g for *E. coli*, *L. innocua* and *S. enterica*, respectively.

Compared to the control all the treatments reduced the *E. coli* population at every sampling time (Fig. 1A). The UV-C dose of 1.0 kJ/m^2 was the treatment that resulted in the lowest population

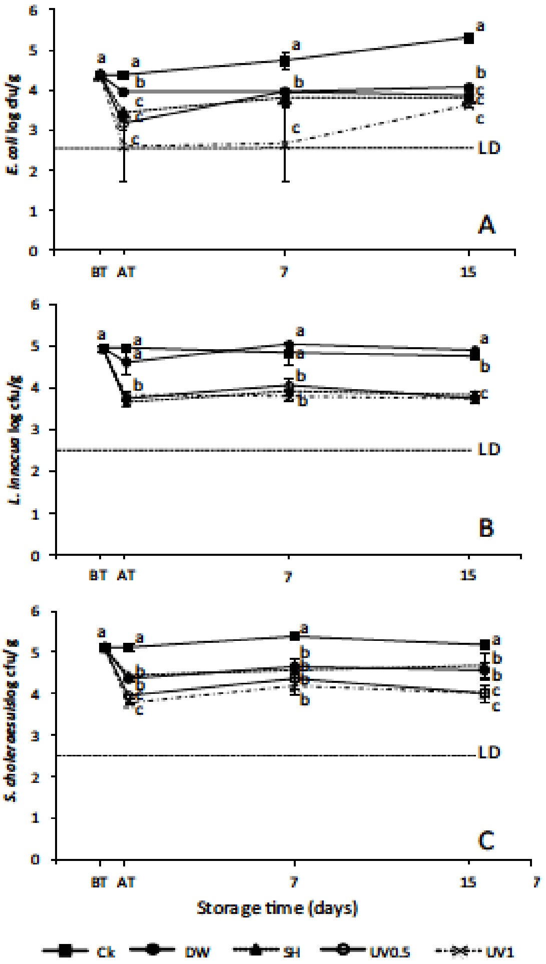

Fig. 1. Growth of (A) *Escherichia coli*, (B) *Listeria innocua* and (C) *Salmonella enterica* inoculated as pure cultures in fresh cut apple pieces stored for 15 days at 4 °C. Ten grams of fruit slices were inoculated by dipping in a 10^7 cfu/mL suspension of a pure culture of each pathogen. For each pathogen different letters indicate significant differences between treatments according to Duncan multiple range test ($P < 0.05\%$). Values are the means of 3 experiments with 4 replicates each and bars indicate standard deviations.

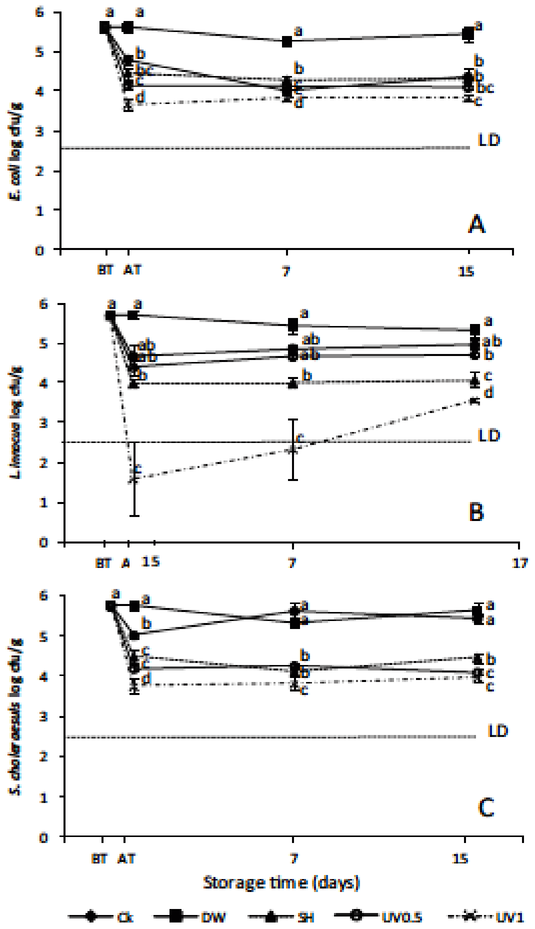

Fig. 2. Growth of (A) *Escherichia coli*, (B) *Listeria innocua* and (C) *Salmonella enterica* inoculated in mixture in fresh cut apple pieces stored for 15 days at 4 °C. Ten grams of fruit slices were inoculated by dipping in a 10^8 cfu/mL suspension mixture of the three pathogens. For each pathogen different letters indicate significant differences between treatments according to Duncan multiple range test ($P < 0.05\%$). Values are the means of 3 experiments with 4 replicates each and bars indicate standard deviations.

of *E. coli* at every sampling and was significantly lower than the *E. coli* population on apple slices treated with SH. After treatment and at day 7, the population was below the detection limit (LD) (2.5 log cfu/g detection limit) in some of the replicates illuminated with the dose of 1.0 kJ/m^2. No significant differences were observed in apple pieces treated with SH or UV-C (0.5 kJ/m^2) both after treatment and during the storage period. DW was the treatment with a higher pathogen recovery level, and no differences were observed on growth rate from after treatment to day 15.

L. innocua reductions obtained by UV-C doses of 0.5, 1.0 kJ/m^2 and SH were no different (around 1.5 log unit) after treatment and during the storage period (Fig. 1B). These three treatments resulted in a higher reduction of the *L. innocua* population than DW. No differences in population of *L. innocua* were observed between Ck and DW at each sampling time.

The highest *S. enterica* population reductions were obtained by both UV-C treatments of 0.5 and 1.0 kJ/m^2 (around 1.5 log unit) at all sample times, except at day 7 were no differences were detected among treatments (Fig. 1C). The *S. enterica* populations were the same on apple slices treated with SH and DW at every sample time.

3.3. Antimicrobial activity of UV-C illumination on fresh-cut apples inoculated with a bacterial cocktail

Fig. 2 represents the survival and growth of *E. coli*, *L. innocua* or *S. enterica* when inoculated in a mixture on fresh-cut apple then treated with UV-C illumination (0.5 and 1.0 kJ/m^2) and stored up 15 days at 4 ± 0.5 °C. The viable cells recovered before treatments on inoculated apple slices were 5.61, 5.69 and 5.78 log cfu/g for *E. coli*, *L. innocua* and *S. enterica*, respectively.

When the three bacteria were inoculated in a mixture, the efficacy of the UV-C treatments to reduce *E. coli* and *S. enterica* populations was higher than washings with SH and DW (Fig. 2A and C). UV-C at a dose of 1.0 kJ/m^2 was the most effective treatment in reducing all pathogens during the storage period. This dose of UV-C after treatment reduced 2.0 log cfu/g the population of *E. coli* and *S. enterica*. *L. innocua* population reach undetectable levels in some replicates (below 2.5 log cfu/g) when apple pieces were illuminated with UV-C at 1.0 kJ/m^2, just after treatment and after 7

Table 2
Determination of quality parameters: color (L^*, a^*, b^*), solid soluble contents (SSC, °Brix) and titratable acidity (TTA, g malic acid/l) in non-inoculated apple slices treated with UV-C illumination.

Color	Treatment (kJ/m$^{2)}$	Days			
		BT	AT	7	15
L^*	0	73.71 ± 0.89 a x	73.91 ± 0.32 a x	70.69 ± 0.73 a y	69.93 ± 0.87 a y
	0.5	73.71 ± 0.89 a x	73.19 ± 0.83 a x	70.23 ± 0.91 a y	70.19 ± 0.95 a y
	1.0	73.71 ± 0.89 a x	73.17 ± 0.53 a x	70.01 ± 0.69 a y	70.01 ± 0.81 a y
a^*	0	−2.36 ± 0.75 a x	−2.38 ± 0.97 a x	−1.18 ± 0.23 a y	−1.07 ± 0.12 a y
	0.5	−2.36 ± 0.75 a x	−2.41 ± 0.65 a x	−1.22 ± 0.52 a y	−1.15 ± 0.34 a y
	1.0	−2.36 ± 0.75 a x	−2.49 ± 0.64 a x	−1.28 ± 0.70 a y	−1.17 ± 0.52 a y
b^*	0	21.78 ± 0.68 a x	21.12 ± 0.74 a x	23.76 ± 0.74 a y	24.13 ± 0.36 a y
	0.5	21.78 ± 0.68 a x	21.19 ± 0.55 a x	24.01 ± 0.55 a y	24.24 ± 0.39 a y
	1.0	21.78 ± 0.68 a x	21.13 ± 0.71 a x	23.99 ± 0.48 a y	24.14 ± 0.79 a y
SSC	0	12.7 ± 0.91 a x	13.1 ± 0.75 a x	12.9 ± 0.88 a x	12.7 ± 0.68 a x
	0.5	12.7 ± 0.91 a x	12.9 ± 0.95 a x	12.0 ± 0.87 a y	12.8 ± 0.74 a x
	1.0	12.7 ± 0.91 a x	13.8 ± 0.78 a x	12.4 ± 0.66 a xy	12.7 ± 0.37 a x
TTA	0	3.51 ± 0.52 a x	3.73 ± 0.31 a x	3.26 ± 0.88 a x	3.22 ± 0.49 a x
	0.5	3.51 ± 0.52 a x	3.53 ± 0.87 a x	3.54 ± 0.45 a x	3.63 ± 0.91 a x
	1.0	3.51 ± 0.52 a x	3.73 ± 0.25 a x	3.38 ± 0.71 a x	3.47 ± 0.21 a x

Each value is the mean ± standard error of the mean of four replicates with 4 evaluations each. Within columns, different letters (a and b) indicate significant differences ($P < 0.05$) between treatments; within lines different letters (x and y) indicate significant differences ($P < 0.05$) between storage days.

days of treatment (Fig. 2B). UV-C at 0.5 kJ/m^2 always resulted in population reductions of the three bacteria equal or superior to 1.0 log cfu/g. In general, no significant differences were observed between this treatment and SH.

3.4. *Quality parameters of apple pieces*

Table 2 shows the L^*, a^*, b^* of color values, solid soluble contents (SSC) and titratable acidity (TTA) values for control (0 kJ/m^2) and both UV-C illuminated (0.5 and 1.0 kJ/m^2) apple slices stored at 4 ± 0.5 °C for 15 days. No differences in color values were observed among treatments, although Hunter L^* values slightly decreased after 7 days of storage in all treatments and then remained stable until day 15. Regarding a^* and b^* values, an increase occurred in all treatments during the first 7 days and then values remained stable.

The SSC and titratable acidity TTA values were not influenced by UV-C treatments or storage period.

4. Discussion

The present study demonstrated that UV-C treatment is effective in significantly reducing the microbial load in fresh-cut apples inoculated with *E. coli*, *L. innocua* and *S. enterica*, and not only after processing but also during storage. This reduction depends on the microorganism and the applied dose of UV-C illumination. Untreated samples had higher total viable counts apart from the treatment applied with only one exception, fresh-cut apple inoculated with a pure culture of *L. innocua* (Fig. 1B). On this case apple pieces washed with DW had the same development throughout storage than untreated apple pieces. The highest reductions were observed on fresh-cut apples treated with UV-C at 1.0 kJ/m^2 just after treatment and throughout storage. In apple pieces inoculated with a pure culture of the pathogens, and treated with UV-C at 1.0 kJ/m^2, population reduction ranged from 1.0 to 2.1 log cfu/g and on those inoculated with a cocktail of the pathogens from 1.4 to 4.1 log cfu/g, just after treatment and at day 7. The highest reduction observed in apple slices inoculated with the mixture of the pathogens could be due not only to the treatments, but also to a competition of nutrients by the pathogens. These results are in agreement with other studies showing that the germicidal effect of UV-C light in fresh-cut fruit and vegetables is usually within 1 and 2 log cycles. Schenk et al. (2012), observed a reduction of 2.5 and 2.2 log of *E. coli* and *L. innocua* in 'William' pear slices at a UV-C dose of 7.5 kJ/m^2. Artés-Hernández et al. (2010) also found that UV-C illumination of fresh-cut watermelon reduces its natural microbial load in 2 log cfu/g and Gómez et al. (2010) reached results of log reduction of *L. innocua*, *E. coli* and *Sacharomyces cerevisiae* ranging from 1 to 1.9 log cycles in apple discs. When comparing UV-C illumination with commercial sanitation SH treatment, the effect on microbial reduction of the application of SH at 100 ppm was in general similar to that obtained with the UV-C dose of 0.5 kJ/m^2.

Schenk et al. (2008) studied the inhibition of some pathogens by UV-C light on pear slices with and without peel. In pear slices without peel for the highest UV-C doses *L. innocua* and *L. monocytogenes* were the most beneficial microorganisms, and in contrast, at lower doses, *E. coli* and *Zygosaccharomyces bailii* were the most beneficial ones. However in pear slices with peel, the survival patterns changed, *E. coli* and *L. innocua* were the most beneficial, showing that there are several factors affecting the behavior of the microorganism submitted to UV-C light.

In our studies, except for *L. innocua* in a mixture, the application of UV-C illumination reduced the initial microbial population in apple pieces, which could not repair induced damage and recover during storage, remaining almost constant during the 15 days of storage. Other authors have found different results, at the beginning of the assay illuminated samples by Manzocco et al. (2011) had reductions of 2 log units until 8 days of storage, but after this period the difference reduced to 1 log cycle.

Yousef and Marth (1988), state that the survival of microorganisms is also affected by the number of illuminated cells. As in our study, Schenk et al. (2008) observed different resistance patterns in experiments using pear slices inoculated with pure cells or with a cocktail of strains.

UV-C illumination can damage microbial DNA and lead to mutations. These mutations can block the transcription and replication of the cell, compromising cellular functions and often leading to cell death. It is known that the amount of this DNA cross-linking is proportional to the UV-C exposure (Allende et al., 2006). It has been reported that the effect of UV-C illumination on the inactivation of microorganisms is dependent on the dose applied, which is defined as intensity multiplied by the exposure time (Sommer et al., 1996). And it is also known that UV-C does not penetrate the tissues, so only the microorganisms on surface will be a target of the UV-C illumination. This phenomenon could explain the reduction values of *L.*

innocua on the apple pieces inoculated with the three pathogens at time 0. The development of this foodborne pathogen after 15 days of storage might be due to the capacity of some microorganisms to repair the DNA damage.

The use of UV-C illumination as eco-innovative disinfection system of fresh-cut apples is in the same direction as other alternative systems and results are in accordance with them. Graça et al. (2011) achieved reductions of pathogens on the order 2 log and higher with the use of electrolyzed water. The application of sodium chlorite and calcium propionate to apple slices was able to inhibit apple browning during storage and reduce *E. coli* population (Guan and Fan, 2010). Abadias et al. (2011) reduced the population of *E. coli* O157:H7, *Salmonella* spp. and *Listeria* spp. in apple plugs with peroxyacetic acid, hydrogen peroxide and N-acetyl-L-cysteine and Alegre et al. (2011) with the application of the probiotic strain *Lactobacillus rhamnosus* GG achieved a reduction of *L. monocytogenes* of 1 log.

Additional analyses were also performed to investigate the effect of UV-C illumination quality properties of fresh-cut apples. Color, solid soluble contents and acidity of fresh-cut apples were not significantly affected by storage up to 15 days independently of the application UV-C illumination during processing. Similar findings were reported from Artés-Hernández et al. (2010) on fresh-cut watermelon and from Manzocco et al. (2011) on fresh-cut melon.

In conclusion the present study demonstrated that UV-C illumination is effective in reducing *E. coli*, *L. innocua* and *S. enterica* on the surface of fresh-cut apple. UV-C illumination at a very low dose of 1.0 kJ/m^2 was found to be more effective on disinfection than a standard sodium chloride solution, indicating that should be considered as an alternative to chlorination in fresh-cut industry. This disinfection technique has also a major advantage because it does not leave residues on the fruit, is safer to the consumer, and could reduce water consumption and minimize waste water discharge rates of the fresh-cut industry.

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Capítulo V

Growth of *Escherichia coli*, *Salmonella enterica* and *Listeria* spp., and their inactivation using ultraviolet energy and electrolyzed water, on 'Rocha' fresh-cut pears

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Submetido

Growth of *Escherichia coli, Salmonella enterica* and *Listeria* spp., and their inactivation using ultraviolet energy and electrolyzed water, on 'Rocha' fresh-cut pears

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Abstract

The present study aimed at evaluating the growth of *Escherichia coli*, *Salmonella enterica*, and *Listeria* spp. and studying the efficacy of Ultraviolet-C (UV-C) illumination, acidic electrolyzed (AEW) and neutral electrolyzed (NEW) waters in the reduction of these bacteria on ‘Rocha’ pear. Fresh-cut pieces were inoculated and incubated at 4-20 °C for 8 days. Inoculated pears were treated with UV-C (2.5-10 kJ/m^2), AEW, NEW and sodium hypochlorite (SH) and microbiological and quality parameters were evaluated. The three bacteria grew on the pear at high growth rates at 12 and 20 °C reaching populations of 8.1-8.6 log cfu/g, in 24 h. At 8 °C the microorganisms increased their populations by at least 1 log cfu/g in three days. At 4 °C adaptation phases of less than 24 h for *Listeria* spp. were measured before exponential growth occurred and the enterobacteria did not grow despite having survived for 8 days. AEW and NEW caused microbial reductions similar to SH, of approximately 1 log cfu/g, while the best UV-C dose (7.5 kJ/m^2) of at least 2.4 log cfu/g. Fresh-cut pears were a good substrate for foodborne bacteria emphasizing the importance of preventing contaminations and cross contaminations. The UV-C was more effective than the chemical decontaminations, as it provided superior microbial reductions without greatly affecting the quality of pears.

Keywords: 'Rocha' fresh-cut pears, *Escherichia coli*, *Salmonella enterica*, *Listeria* spp., Ultraviolet-C, Electrolyzed water

1. Introduction

The safety and the increase of shelf-life of minimally processed foods are two major challenges for the industry as fresh products may contain high microbial levels after harvesting and can be easily contaminated with foodborne microorganisms during the processing (Graça et al 2015, 2017; Ölmez and Kretzschmar, 2009; Parish et al., 2003; Ramos et al., 2013).

The natural microbiota of raw fruits and vegetables is usually nonpathogenic for humans and is present at the time of consumption. However, during primary production and processing, the food can be contaminated with pathogens from human, animal or environmental sources (Brandl, 2006). Fresh fruit products (apple juices, tomatoes, watermelon, mango, cantaloupe, berries) have been responsible for outbreaks caused by pathogenic bacteria such as *Escherichia coli* O157:H7, *Salmonella enterica* and *Listeria monocytogenes* (Ölmez and Kretzschmar, 2009; Parish et al., 2003; Ramos et al., 2013). The growth of pathogens on food during distribution/storage is thought to be determinant to most outbreaks (Codex Alimentarius Commission, 1999) and several studies have demonstrated the capacity of pathogenic bacteria to survive and/or grow at different temperatures in minimally processed fruits (Abadias et al., 2012; Alegre et al., 2010a, 2010b; Dingman, 2000; Lourenço et al., 2012, Santo et al., 2016). Moreover, different produce differ in the ability to support the growth of bacteria as reported for *L. monocytogenes* (Hoelzer et al., 2012). The processing operations inherent to the minimal processing which include cutting, dicing, washing, decontamination and packaging are determinant to the contamination levels and for the microbial growth behavior. Operations such as cutting and dicing increase the availability of nutrients and contribute to the dissemination of microorganisms and their growth. Additionally, the capacity of microorganisms to produce biofilms on fresh produce may enhance their survival and growth and enable the bacteria to persist and withstand washing and antimicrobial treatments. *Salmonella* Typhimurium embedded in a biofilm matrix resisted sodium hypochlorite (NaOCl) at concentrations above 500 mg/L, while planktonic cells were sensitive to less than 50 mg/L (Scher et al., 2005).

Sodium hypochlorite (50 to 200 mg/L, during 1-2 minutes) is the most widespread disinfectant applied in the fresh-cut industry, although it can cause problems to man and the environment due to the generation of potentially harmful by-products such as gases, trihalomethanes and chloramines. Additionally, its efficacy is dependent on pH, organic material and the physiologic state of microorganisms, and its use is prohibited in some European countries. As a consequence, alternative chemical and physical decontamination methods are studied (Beuchat, 1998; Ramos et al., 2013).

Short wave Ultraviolet-C (UV-C) illumination and electrolyzed water (EW) are two non-thermal decontamination technologies that have been tested as alternatives to chlorine. Different studies have reported that UV-C light at 254 nm in doses from 0.5 to 20 kJ/m^2 reduces the number of microorganisms, thus contributing to the extension of shelf-life while maintaining and/or improving the overall safety and quality of fresh-cut fruit (Bintsis et al., 2000). The main injuries of UV-C on microorganisms, especially on *E. coli,* result from membrane alterations on phospholipids, secondary structures of proteins, and polysaccharides and changes on structures of DNA/RNA (Syamaladevi et al., 2013). This technique was successfully applied to reduce microbial contamination and/or to extend shelf-life in mango and pineapple (George et al., 2015), watermelon (Artés-Hernández et al., 2010), kiwifruit (Beirão da Costa et al., 2014), apples (Graça et al., 2013), apricot (Yun et al., 2013) and melon (Manzocco et al., 2011). Moreover, the UV-C illumination has been associated to the enhancement of antioxidant activity measured in mango and pineapple (George et al., 2015) and in watermelon (Artés-Hernández et al., 2010), to the increase of peroxidase activity in cantaloupe (Lamikanra et al., 2005), to the induction of the production of anthocyanins and stilbenoids (Ramos et al., 2013) and the promotion of enzymatic stability in fresh-cut fruit through the inactivation of pectate lyases (Manzocco et al., 2009a) and polyphenoloxidases (Manzocco et al., 2009b) in apples. The major advantages of UV-C illumination reside in the fact that it is a dry cold process that does not require expensive or high energy consuming equipment, involve extensive safety equipment, leave toxic residues, has broad-spectrum microbicidal activity and is relatively inexpensive (Artés et al., 2009; Guerrero-Beltrán and Barbosa-Cánovas, 2004; Ramos et al., 2013).

EW has been reported to have a great microbicidal activity against several pathogenic and spoilage microorganisms and has also the advantage of neutralizing harmful substances such as cyanides and ammonium (Huang et al., 2008; Ramos et al., 2013). It is produced through the electrolysis of a sodium chloride solution in electrolytic cells where two types of EW can be formed: acidic electrolyzed water (AEW), produced at the anode, and neutral electrolyzed water (NEW) produced at the cathode. AEW has low pH (2-4), high oxidation-reduction power (ORP) (> 1000 mV) and contains oxygen gas, chlorine gas, hypochlorite ion, hypochlorous acid and hydrochloric acid. NEW is characterized by pH values of 5 to 8.5 and ORP values of 500 to 700 mV and contains hydrogen gas and sodium hydroxide (Huang et al., 2008). Although the mode of action of EW is not clearly understood its antimicrobial activity may be related to the disruption it causes in the cell wall of bacteria (Osafune et al., 2006) and to the high oxidizing potential of hypochlorous acid producing hydroxyl radicals ($^{\bullet}$OH) which act on cells and its components (proteins, nucleic acids) (Huang et al., 2008). Electrolyzed water has been used as a disinfectant for food processing equipment and has also been successfully applied to decontaminate fruits and vegetables, among other food. Its application contributes to the reduction of the microbial load on blueberries (Kim and Hung, 2012), tomatoes and lettuce (Pangloli and Hung, 2011), broccoli (Martinez-Hernandez et al., 2015), lettuce, carrot and endive (Abadias et al., 2008) and cilantro (Wang et al., 2004). In fresh-cut apple, both AEW and NEW revealed microbiocidal activity on *E. coli*, *L. innocua* and *S. enterica* as described by Graça et al., (2011). The main advantages of EW are its broad-spectrum microbicidal activity, its safety, as it is not corrosive to humans' health (skin, mucous membranes), is less reactive with organic material and has a less adverse impact on the environment (Huang et al., 2008).

'Rocha' pear (*Pyrus communis* L. cv Rocha) is a Portuguese variety being recognized as a Protected Denomination Origin (PDO) fruit. Its production reached 195,000 tons in 2013, accounting for 95 % of the national pear production from which about 30 % was exported. Due to its characteristics, namely flavor and texture, recently it began to be marketed as minimally processed fruit in restaurants, supermarkets and on airline travel caterings. Since no information is available on the capacity of foodborne pathogens to grow on 'Rocha' pear tissues and on the effect of decontamination technologies on fresh-cut pieces of this fruit, the aim of the present work was to study the growth of *E. coli*, *S. enterica* and *Listeria* spp. on minimally processed 'Rocha' pear at different temperatures and evaluate the efficacy of UV-C illumination, acidic and neutral electrolyzed water on reducing the mentioned bacteria population, inoculated individually and in a mixture, in fresh-cut pears.

2. Methods

2.1. Pear preparation

The ‘Rocha’ (cv) pears used in the present study were purchased in an orchard and stored at 0.5±0.5 °C before processing. Pears were washed in running tap water and surface disinfected by dipping and scrubbing in a sodium hypochlorite solution (0.5 %) during 30 s. After drying at room temperature, pears were aseptically cut in pieces of 1 g each (1 cm long and radius 0.6 cm obtained with a sterile cork borer), without core tissue and skin. Pieces of 10 g each without core tissue and with the skin were prepared, using a cutting instrument, to perform the decontaminations and evaluate the quality of the fruit.

2.2. Microorganisms and preparation of inocula

The bacterial species used in the present work were *Escherichia coli* (the non-toxicogenic strain of *E. coli* O157:H7 NCTC 12900, *E. coli* ATCC 25922 and *E. coli* ATCC 10536), *Listeria innocua* CECT-910, *L. monocytogenes* C897 (Faleiro et al., 2003) and *Salmonella enterica* (subsp. *enterica* Michigan ATCC BAA-709 and *S.* Typhimurium ATCC 14029). The bacteria were stored at -80 °C and maintained on Tryptone Soy Agar (TSA) (Oxoid, Hampshire, UK) at 4±1 °C. Bacterial inocula used to contaminate the fruit, were cultivated on TSA and incubated during 24±2 h at 37±1 °C. Then, they were sub-cultured in 50 mL of Tryptic Soy Broth (TSB) (Biokar Diagnostics, Allonne, France) following an orbital incubation (VWR, Incubating Mini Shaker, USA) at 150 rpm at 37±1 °C. After 24 h, the bacterial cells were recovered by centrifugation at 8000 rpm for 15 min (Heraeus, Multifuge 1 L-R, Germany) and the pellet was resuspended in 50 mL of sterile saline peptone [8.5 g/L NaCl (Panreac, Barcelona, Spain) and 1 g/L peptone (Biokar)]. These suspensions were used as inocula of fresh-cut pear, after an adjustment of its concentration to 10^7 cfu/mL according to a standard curve, measuring the transmittance at 420 nm in a spectrophotometer (Spectrophotometer UV–Vis, 175 Shimadzu-UV160, USA). The concentrations of bacterial suspensions used as inocula were confirmed using the Miles and Misra (1938) surface colony count method. Drops of 20 µL of ten-fold dilutions were released in triplicate onto the surface of the TSA medium and plates were incubated at 37±1 °C for 24±2 h.

2.3. Growth of *E. coli*, *S. enterica* and *Listeria* spp. on fresh-cut pears at different temperatures

The growth of *E. coli*, *S. enterica* and *Listeria* spp. on fresh-cut pears at different temperatures was performed on 1 g pear pieces previously prepared as described above. Pear portions were submerged in 10^7 cfu/mL suspensions of *E. coli*, *S. enterica* and *Listeria* spp. separately, during 3 min at 150 rpm in an orbital shaker. After drying in a laminar flow hood (Bioquell, Microflow, UK) during 30 min, samples were divided in 7 sets. Each set was divided in 4 other groups containing 4 pear pieces each. One set was analyzed straightaway. The other 6 batches were packed in **biaxially-oriented polypropylene (BOPP)** (0.030 mm thick) bags and each batch was stored at four different temperatures: 4±0.5 °C, 8±0.5 °C, 12±0.5 °C and 20±0.5 °C.

The population of the three different bacteria was enumerated, individually, on the fresh-cut pear samples at days 0, 1, 2, 3, 6 and 8, after the storage. The inoculated pear portions (1 g) were transferred into sterile Stomacher bags, mixed with 9 mL of sterile saline peptone and homogenized in a Stomacher (Model 400 Circulator, Seward, Norfolk, England) during 2 min. Homogenates were serially diluted in saline peptone and aliquots of 20 µL were plated in triplicate on the surface of Sorbitol MacConkey agar (Biokar Diagnostics) to count the number of *E. coli*, on Palcam agar (Biokar Diagnostics) to evaluate the population of *Listeria* spp. and on Hektoen agar (Biokar Diagnostics) to enumerate *S. enterica*. The evaluation of the microbial populations was performed with the Miles and Misra method (Miles and Misra, 1938). Plates were incubated at 37±1 °C for 24±2 h (*E. coli* and *S. enterica*) or for 48±2 h (*Listeria* spp.). Colonies were counted and the results expressed as colony forming units (cfu) per gram of pears. In each sampling point, four replications were performed and the experiments were repeated twice. The specific growth rates (day^{-1}), adaptation phases (Lag) (day) and final microbial population (Final value) (log cfu/g) were calculated using the DMFit modeling tool (http://modelling.combase.cc) (Barany and Roberts, 1994).

2.4. UV-C treatment

The UV-C treatments were performed in a chamber (100 cm x 100 cm x 50 cm) equipped with two sets of five unfiltered germicidal emitting lamps (Philips, TUV 25W G25 T8 Longlife). One set of lamps was placed horizontally on the top and the other one on the bottom of the radiation cabinet. The fresh-cut pears were placed on a net positioned midway between the UV-C lamps. The walls of the cabinet enhanced a homogeneous dispersion of the emitted light to allow illumination of almost the whole food surfaces. The UV-C radiation intensity of the lamps was measured with a radiometer (UVX Radiometer, UVP. Inc, USA) placed at the same distance as the commodities (15 cm) and calculated as a mean of 20 readings in different places taken at each side of the net. The intensity of light was kept constant and the applied doses varied by modifying the exposure time. The UV-C doses selected to use as decontamination treatments on fresh-cut pears were 2.5, 5, 7.5 and 10 kJ/m^2 and will be referred to as UV2.5, UV5, UV7.5 and UV10, respectively (in the images, tables and text).

2.4. Electrolyzed water

Acidic electrolyzed water (AEW) and neutral electrolyzed water (NEW) were produced with an electrolyzed water (EW) generator (Envirolyte EL-400, Envirolyte Industries International Ltd., Estonia) when a saturated sodium chloride solution was pumped into the equipment with the current set at 20–23 A, according to the instructions of the manufacturer. AEW and NEW were collected in flasks and kept at 4 °C until use (no more than one day). Solutions of AEW and NEW were prepared at 100 mg/L of free chlorine by diluting with distilled water previous to its application on the fruit.

UV-C illumination treatments and AEW and NEW washings were compared with distilled water (DW) and sodium hypochlorite (SH) solutions at 100 mg/L free chlorine. SH solutions were prepared by diluting a 4 % sodium hypochlorite solution (AppliChem, Darmstadt, Germany) with distilled water. All solutions were stored at 4 °C and used within 1 h. The properties of each solution such as ORP, pH and free chlorine concentration were measured. ORP and pH were measured with a pH-meter (Model GLP-21, Crison, Spain), using an ORP electrode (Crison 52-61) and a pH electrode (Crison 52-02), respectively. Free chlorine concentrations were determined using a free and total chlorine photometer (HANNA Instruments, model HI9133, Woonsocket, RI, USA). The AEW used in the decontamination treatments had a pH of 2.90 (±0.03), a ORP of 1121 (±3) mV and a free chlorine of 99 (±2) mg/L. The NEW applied in the fresh-cut pear was characterized by a pH of 8.20±0.11, a ORP of 754±5 mV and contained 102±2 mg/L of free chlorine.

2.5. Inactivation of *E. coli, S. enterica* and *Listeria* spp. (individually and in a mixture) on fresh-cut pears using UV-C illumination and AEW and NEW

Fresh-cut pear pieces were immersed in a 10^7 cfu/mL suspension of *E. coli*, *S. enterica*, *Listeria* spp. individually, during 3 min with 150 rpm orbital agitation. Inoculated samples were air-dried in a laminar flow hood during 30 min before the application of the treatments.

Inoculated pear pieces were divided into 9 batches of 4 pieces each. Four batches were submitted to UV-C light treatment of 2.5, 5, 7.5 and 10 kJ/m^2, each. Two of the batches were used to study the effect of washings with AEW and NEW as decontaminants and another two sets of fruits were treated with SH solution and with DW. The washings treatments (AEW, NEW, SH and DW) occurred by dipping the fruits in flasks containing 500 mL of the treating solutions, during 5 min in agitation (150 rpm) in an orbital agitator. After the application of the treatment solutions, pear pieces were drained and rinsed with cold distilled water for 3 min at 150 rpm in an orbital shaker. Then, these four batches were left to dry in a laminar flow hood for 30 min.

The last inoculated batch of fresh-cut pear was not submitted to any decontamination treatment and was used as control.

In the case of fresh-cut pears inoculated with a bacterial mixture, *E. coli*, *S. enterica* and *Listeria* spp. were prepared as previously described to achieve a final concentration of 10^8 cfu/mL of each bacterium. The quantification of each microorganism was confirmed using the Miles and Misra method (1938), plating 20 µL drops of diluted cultures on Sorbitol MacConkey Agar for *E. coli*, and Hektoen Agar for *S. enterica* (incubation at 37±1 °C for 24±2 h) and on Palcam Agar for *Listeria* spp. (incubation at 37±1 °C for 48±2 h). Samples of pear pieces were inoculated by dipping into 500 mL of a mixture of the three bacteria and left to dry. Afterwards, EW and UV-C treatments, as well as SH and DW, were applied as previously described. Inoculated, but untreated samples were used as a control.

The evaluation of the population of each foodborne bacteria was determined in the pear samples after drying for 30 min. For each decontamination treatment, 10 g of pear pieces were transferred into sterile Stomacher bags and mixed with 90 mL of sterile saline peptone following a homogenization in a Stomacher, during 2 min, as previously described. Serial dilutions in saline peptone were made and 20 µL drops, in triplicate, were plated on the surface of the TSA medium using the Miles and Misra method (1938). Colonies were counted after incubation during 24±2 h at 37 °C, and the results expressed as log cfu/g of pears. For each treatment condition four replications were performed and the experiment was repeated twice.

2.6. Effect of UV-C illumination and AEW and NEW on the quality parameters of fresh-cut pear

The effects of UV-C illumination (2.5, 5, 7.5 and 10 kJ/m^2), AEW and NEW (100 mg/L of free chlorine), SH (100 mg/L of free chlorine) and distilled water (DW) on the quality parameters (color, soluble solid content, titratable acidity, pH and firmness) of fresh-cut pear were also studied. The quality parameters were measured, in triplicate, in pear pieces decontaminated with each treatment, 4 hours after the treatments when the fruit pieces submitted to washings were dried. Results were compared with determinations performed with untreated fresh-cut pear immediately after cutting (AC) and 4 hours after cutting, used as control (CK).

Surface color of pear pieces was evaluated with a CR-300 Minolta chromameter (Minolta, Inc., Tokyo, Japan), standardized against a white tile, using the CIE *L**, *a**, *b** parameters. The Hue angle was calculated from averaged *a** and *b**.

The soluble solid content (°Brix) (SSC) of fresh-cut pears was measured using a refractometer (Atago Co. Ltd. Tokyo, Japan) in the juice extracted from the pear pieces.

Titratable acidity (TA) was measured in 10 mL of pear juice dilute in 10 mL of distilled water and titrated with 0.1 N of NaOH (Merck, Darmstadt, Germany) to a pH value of 8.2. Results were calculated as g of malic acid per liter.

Firmness was determined using a texture analyzer (Chatillon, Chatillon Force TCD200, Digital Force Gauge Dfis 50 penetrometer, USA) with a 8 mm diameter plunger that penetrated 7 mm. Firmness was expressed in Newton (N).

2.7. Statistical analyses

The values of reduction in bacteria on pear pieces were calculated by subtracting the population of inoculated but untreated pears from the microbial population after treatment in the same storage conditions. Values represent the means of 2 different experiments, with 4 replicates per treatment per experiment. The quality parameters were determined in triplicate in samples decontaminated with each treatment. Data were subjected to analysis of variance and Duncan's multiple range tests using SPSS v.20.0 software (SPSS Inc., USA). Significant differences in reduction values were established by the least significant difference at the 0.05 level of significance.

3. Results and Discussion

3.1. Growth of *E. coli, S. enterica* and *Listeria* spp. on fresh-cut pears

The survival and growth of *E. coli* (Fig. 1A), *S. enterica* (Fig. 1B) and *Listeria* spp. (Fig. 1C) inoculated on fresh-cut 'Rocha' pears, at different temperatures (4, 8, 12 and 20 °C) during a period of eight days are represented in Fig. 1. At 20 °C the population of the three foodborne pathogens increased exponentially during approximately the first day, with maximum specific growth rates of 2.98±0.258, 2.7±0.322 and 3.1±0.296 day^{-1}, for *E. coli*, *S. enterica* and *Listeria* spp., respectively. At 12 °C a similar behavior was observed for the three microorganisms but with maximum specific growth rates slightly lower of 1.9±0.193, 2.2±0.23, 2.6±0.636 day^{-1}, respectively. After the exponential growth a stationary phase occurred until the end of the assays. An increase of initial viable populations, recovered from inoculated fresh-cut pears, of 6.0-6.4 log cfu/g to 8.1-8.6 log cfu/g, at the end of the study was observed.

At 8 °C, *E. coli* and *S. enterica* were able to grow exponentially during approximately 3 days at maximum specific growth rates of 0.37±0.043 and 0.66±0.127 day^{-1}, respectively, although more slowly than the temperatures of 12 and 20 °C. Then, a stationary phase growth was observed until the 8th day when final populations of 7.9±0.074 and 7.6±0.124 log cfu/g, respectively, were counted. Regarding *Listeria* sp., an adaptation phase of 0.58±0.279 day was estimated, which was followed by exponential growth at a rate of 0.89±0.113 day^{-1}, reaching the stationary phase, approximately, after 3 days. Counts of the *Listeria* population increased from 6.3±0.040 log cfu/g, at the beginning, to maximum values of 9.2±0.054 log cfu/g of pear, at end of the experiment.

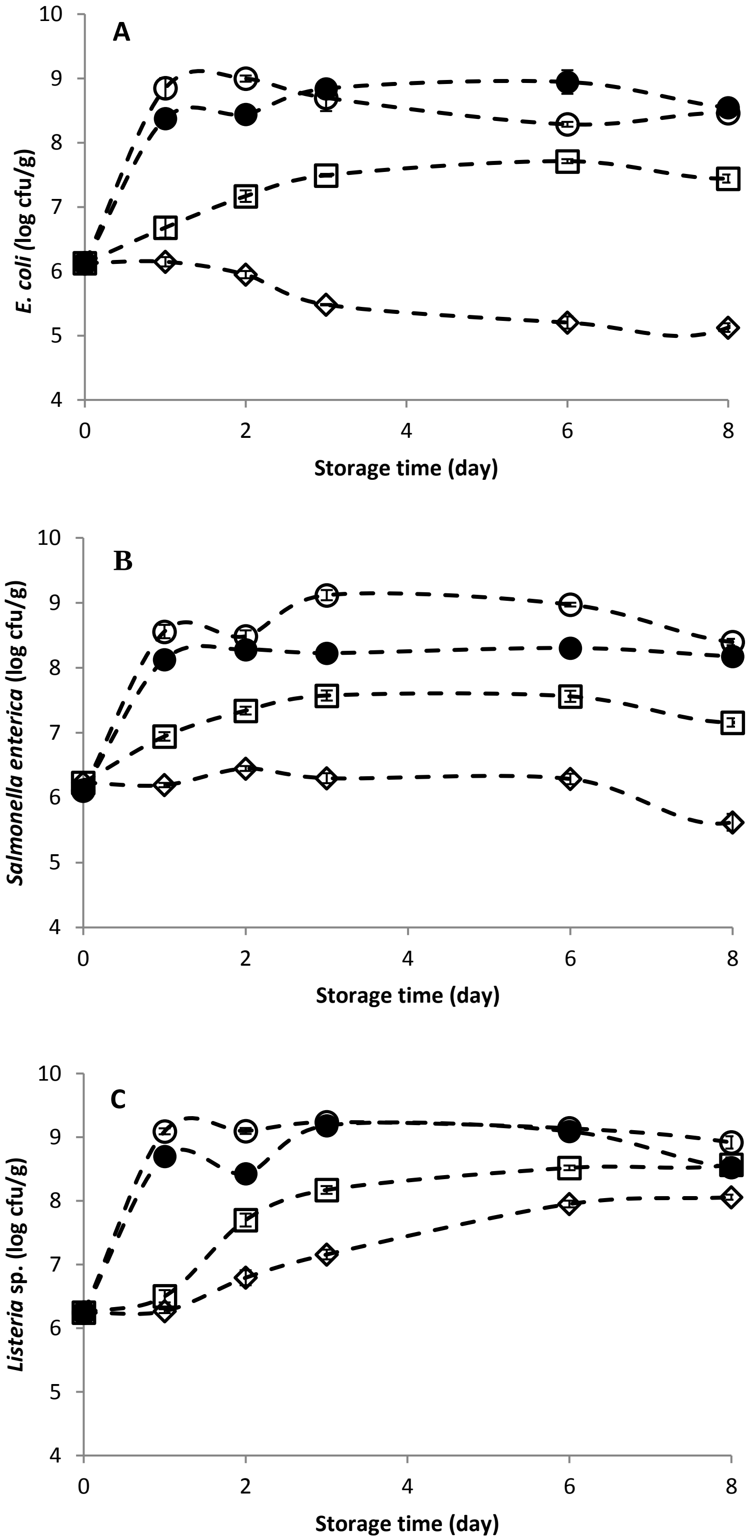

Fig. 1. Growth of inoculated bacteria in pear pieces stored for 8 d at 4°C, 8°C, 12°C and 20°C. (A) *Escherichia coli*; (B) *Salmonella enterica*; (C) *Listeria* sp.. Values are the means of 2 experiments with 4 replicates each and bars indicate standard error. (◊ 4 °C; □ 8 °C; ● 12 °C; ○ 20 °C).

At 4 °C the population of *E. coli* and *S. enterica* remained almost unchanged during the period studied, after the inoculation moment, or slowly declined. In the case of *E. coli* a death rate of -0.35±0.13 day $^{-1}$ was calculated. In regards to the growth of *Listeria* spp. in fresh-cut pears at 4 °C, an adaptation phase of less than 24 h was estimated followed by an exponential growth at a rate of 0.38±0.0567 day^{-1} reaching a population of 8.2±0.102 log cfu/g.

The results described for pear are similar to previous research regarding the growth of foodborne pathogens in cut fruit at the temperatures tested. For example, *E. coli* O157:H7, *S. enterica* and *L. innocua* were able to growth exponentially at temperatures of 20 and 25 °C on fresh-cut peaches of different varieties (Alegre et al., 2010b) and on fresh-cut apples 'Golden delicious' (Alegre et al., 2010a). At 10 °C these microorganisms were able to grow on the fruits reaching lower populations while at 5 °C, only *L. innocua* was able to multiply. *E. coli* O157:H7 also showed an exponential growth in minimally processed melon at 25 °C but was unable to grow on pineapple at 25 and 5 °C (Abadias et al., 2012). Strawn and Danyluk (2010) observed a similar behavior of *E. coli* O157:H7 and *S. enterica* on cut papayas and mangos at 23 °C. At 12 °C only *Salmonella* grew on both fruits and *E. coli* was only able to growth on papayas. The same authors observe that both enterobacteria did not grow on the fruits at 4 °C but were able to survive during 28 days.

The differences in the growing capacity of bacteria on the fruits may be explained by intrinsic characteristics of the fruits' tissues, including pH, composition, presence/absence of inhibitor compounds and by the physiologic capacity of the different microbial species to adapt to eventual stressful conditions. In the case of peaches, for example, the highest populations of foodborne bacteria registered were obtained in the varieties with the highest pH values (4.12 and 4.73) (Alegre et al., 2010b) and on fresh-cut strawberries (pH 3.6-3.8) Flessa et al. (2005) and Knudsen et al. (2001) reported that *E. coli*, *S. enterica* and *L. monocytogenes* were not able to grow.

The results presented indicate that fresh-cut pears are a good substrate for the three pathogens to survive and grow at temperatures above 8 °C while at 4 °C, only *Listeria* spp. was able to grow after 24 h adaptation phase. Fresh-cut pear has a pH tissue value of 5.28 which is slightly acidic for a fruit and has a low titratable acidity of 1.3 g malic acid/g, when compared to other fruits (peaches- 4.1-8.9 g malic acid/l; apples-2.16-8.2 g malic acid/l).

Storage temperature is one of the main factors regulating the microbial growth in the food matrices. *Listeria* is a psychrotrophic microorganism and when at refrigeration temperatures induces a complex mechanism of adaptation, the “cold shock response”, that allows it to rapidly adapt and multiply reaching dangerous populations enough to cause disease during the shelf-life of food (Melo et al., 2015). On the other hand, many microorganisms in environments where pH is lower than optimal developed a number of alterations, involving the activation of a number of genes. For example, cells may alter the external pH value by expressing enzymes whose function is to raise external pH, such as lysine decarboxylase, in *Salmonella*, which converts lysine to cadaverine, an alkaline substance, arginine decarboxylase in *E. coli* (Beales, 2004) and arginine deiminase in *L. monocytogenes* (Melo et al., 2015). Exposure to mildly acidic conditions induces tolerance mechanisms, such as the acid tolerance response (ATR) described in the foodborne microorganisms *S. enterica* and *E. coli* (Foster, 2001) and *L. monocytogenes* (Melo et al., 2015). These mechanisms, among others, enable the bacteria to survive on food products such as fruits, with a pH lower than the microbial optimal pH, and protect them from subsequently more severe pH/acid conditions. Microorganisms may evolve to being able to rapidly adapt and tolerate/resist a particular stress. This adaptation or resistance will allow the survival and growth of foodborne microorganisms, thus having great implications on the safety of food products, such as acidic food stored at low temperatures.

3.2. Inactivation of *E. coli, S. enterica* and *Listeria* spp. (individually and in a mixture) on fresh-cut pears using UV-C illumination and AEW and NEW

The antimicrobial activity of UV-C illumination at different doses (2.5, 5, 7.5 and 10 kJ/m^2) and electrolyzed water (AEW and NEW) (100 mg/L of free chlorine), on fresh-cut pears inoculated with single cultures of *E. coli, S. enterica* and *Listeria* spp. is represented in Fig. 2 and with a mixture of the three groups of microorganisms, in Fig. 3. The results were compared with fresh-cut fruit treated with SH solution (100 mg/L of free chlorine) and distilled water (DW).

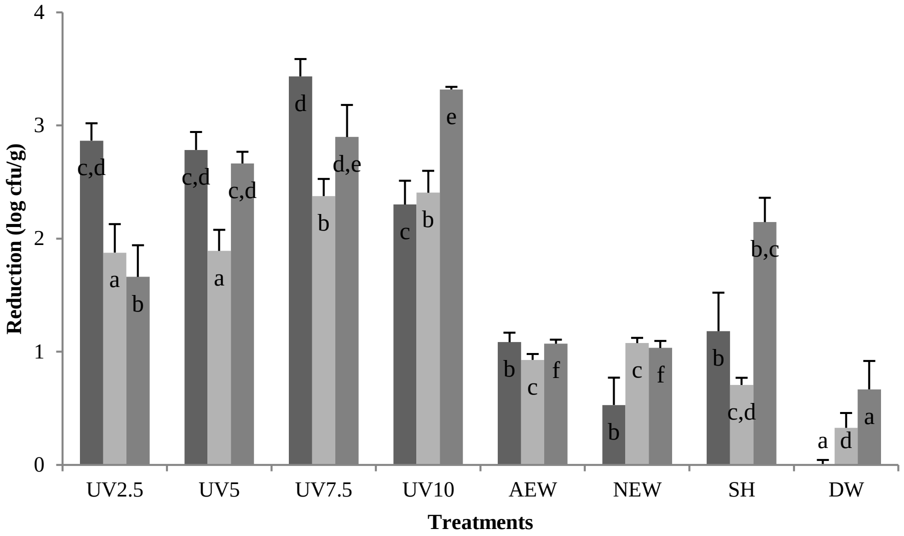

Fig. 2. Reduction of *E. coli*, *S. enterica* and *Listeria* spp. (individually) after treating pears slices with UV-C illumination, acidic electrolyzed water (AEW), neutral electrolyzed water (NEW), sodium hypochlorite (SH) (100 mg/L of free chlorine) and with distilled water (DW). For each pathogen, columns with different letters indicate significant differences between treatments using Duncan multiple range test ($P < 0.05\%$). Values are the means of 2 experiments with 4 replicates each and bars indicate standard errors. (■ *E. coli*; ■ *S. enterica;* ■ *Listeria* spp.).

The exposure of pear pieces to the different doses of UV-C illumination and EW, as decontaminants, induced reductions in the populations of the three foodborne pathogens studied. In the case of *E. coli* population (in a single culture) the reductions obtained ranged from 2.3 log cfu/g to 3.4 log cfu/g after the application of UV10 and UV7.5, respectively ($p<0.05$) (Fig. 2). When *E. coli* was inoculated in the pear with a mixture of species, the most efficient treatment was also UV7.5 resulting in the highest reduction values of *E. coli* population of 3.2 log cfu/g (Fig. 3). None of the UV-C treatments resulted in microbial reductions inferior to 1.97 log cfu/g. Regarding EW washings, microbial decreases values of 0.53 to 1.1 log cfu/g were achieved and no significant differences among the bacterial population drops obtained in samples washed with AEW, NEW or SH were observed ($p>0.05$), whether in a single culture or in a cocktail (Fig. 2 and Fig. 3). The microbial reductions obtained with decontaminations of AEW and NEW showed no differences from the results achieved with washings of SH solutions ($p>0.05$).

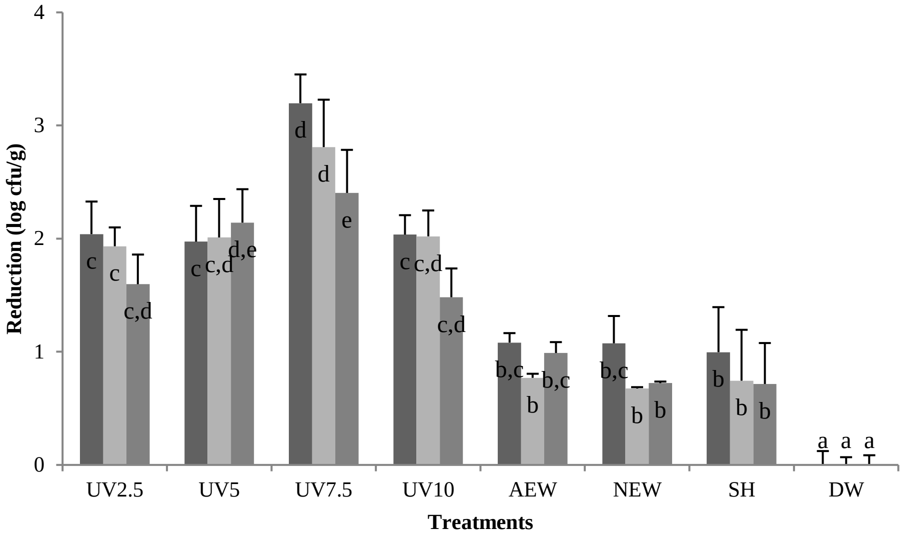

Fig. 3. Reduction of *E. coli*, *S. enterica* and *Listeria* spp. (mixture of the 3 bacteria) after treating pears slices with UV-C illumination, acidic electrolyzed water (AEW), neutral electrolyzed water (NEW), sodium hypochlorite (SH) (100 mg/L of free chlorine), and with distilled water (DW). For each pathogen, columns with different letters indicate significant differences between treatments using Duncan multiple range test (P < 0.05%). Values are the means of 2 experiments with 4 replicates each and bars indicate standard errors (■ *E. coli*; ■ *S. enterica;* ■ *Listeria* spp.).

The application of UV-C illumination on fresh-cut pear inoculated with *S. enterica* in a single culture led to the highest reductions of this microorganism when doses of UV10 and UV7.5 were applied with values of 2.4 and 2.4 log cfu/g, respectively and no statistical differences were found between them (p>0.05) (Fig. 2). In the mixed culture, the UV7.5 was also the treatment that allowed the higher reduction values (2.8 log cfu/g) for *S. enterica* (Fig. 3). None of the UV-C treatments resulted in the reduction level of *S. enterica* inferior to 1.9 log cfu/g. Washing the contaminated pears with AEW and NEW caused a decrease in the levels of *S. enterica* population of 0.92 and 1.1 log cfu/g in a single culture (Fig. 2) and of 0.76 and 0.67 log cfu/g in a mixed culture (Fig. 3). In both cases, the results obtained in the decontaminations with AEW and NEW showed no differences from the disinfections performed with SH (p>0.05). The washing with DW was the treatment that resulted in lowest reduction values, of *E. coli, S. enterica* and *Listeria* spp. populations on the fresh-cut pears.

With regards to *Listeria* spp. in a single culture inoculation, the highest reductions were achieved when the UV10 treatment (3.3 log cfu/g) and UV7.5 (2.9 log cfu/g) were applied and no statistical differences between these results were detected (p>0.05). The lowest microbial reduction of 1.7 log cfu/g was caused by UV2.5 (Fig. 2). When the fresh-cut pears were inoculated with a mixture of the three pathogens, the highest reduction (2.4 log cfu/g) of *Listeria* spp. was obtained with the UV7.5 treated samples, although there were no statistical differences from UV5 treated pears (2.1 log cfu/g) ($p>0.05$). The lowest reductions were observed with the UV10 (1.5 log cfu/g) (Fig. 3). Concerning the utilization of EW as a decontaminant, no significant differences were observed among the microbial reductions achieved in the pear samples washed with AEW and NEW, which caused a decrease in *Listeria* spp. values of 1.1 and 1.03 log cfu/g, in a single culture (Fig. 2), and 1.1 and 0.92 log cfu/g in the mixture (Fig. 3), respectively. Washing with SH resulted in higher reduction values of *Listeria* spp. population than those caused by the utilization of AEW and NEW, when inoculated in a single culture but not in a mixed culture.

According to the results obtained, *E. coli*, *S. enterica* and *Listeria* spp. populations were significantly reduced in fresh-cut pear by UV-C and EW treatments. The UV7.5 appeared to be the most efficient decontamination method, as its application resulted in the decreasing of the three foodborne populations of pathogens higher than 2.4 log cfu/g when inoculated in a single or in a mixed culture. Additionally, as can be observed in Fig. 2 and Fig. 3 the application of higher doses of UV-C than UV7.5 did not always result in higher microbial load reductions. The UV-C decontaminations were more effective than the ones performed with SH which resulted in reductions less than 1 log cfu/g with exception of *E. coli* and *Listeria* sp. (when inoculated in a single cultures). Regarding EW decontaminations, the level of microbial reductions achieved did not exceed 1.1 log cfu/g. EW decontaminations resulted in lower microbial reductions compared to those obtained when the UV-C was applied, although they were not significantly different from the decontaminations performed with SH. Previous studies conducted by Syamaladevi et al. (2013) to evaluate the effect of UV-C on pear (Fresh D’Anjou cv) decontamination achieved reduction values of *E. coli* population of 3.7 log cfu/g on the surface of intact fruits and 3.1 log cfu/g on wounded fruits using UV-C illumination at the dose 7.56 kJ/m^2. Jemmi et al. (2014) observed that the dose 6.22 kJ/m^2 was more effective than 8.3 kJ/m^2 in reducing yeasts and molds and the total mesophilic on palm dates. The effectiveness of UV-C illumination in the inactivation of *E. coli*, *L. innocua* and *S. enterica* were also observed on apples (1.0 kJ/m^2) (Graça e al.,

2013) and of *E. coli* O157:H7 and different serotypes of *S. enterica* in apricots (Yun et al., 2013). Yaun et al. (2004) used UV-C light to inactivate the population of *E. coli* and *S. enterica* on lettuce, tomato and apple surfaces and observed that the UV-C was more effective against these bacteria than SH (20-320 ppm). When comparing UV-C with EW decontaminations, Kim and Hung (2012) reported that UV-C treatments were more effective than EW inactivating *E. coli* O157:H7 in blueberries. In the present study, decontamination of pears with AEW, NEW and SH were less effective on the bacterial reduction than was UV-C illumination. This is in agreement with the results presented by Kim and Hung (2012). Nevertheless, unlike the results of Graça et al. (2011) AEW was not more efficient than NEW in reducing the level of *E. coli*, *S. enterica*, *L. innocua* in pear as it was in apple. The reaction of chlorine with the organic components of cut fruits has been used to explain its low activity due to the lowering of its effective concentration before damaging microorganisms (Graça et al., 2011). The fact that AEW and NEW showed equal disinfection efficacy than SH indicates that these techniques can be used as an alternative to SH, as they are safer and do not present great health/environmental problems compared to NaClO. Additionally, the effect of the different decontaminations on pear pieces was not affected by the total population size since the microbial reductions achieved on the samples inoculated with a combination of the three groups of microorganisms was similar to that inoculated with only one group of bacteria. This has been reported in other studies, such as in apples (Graça et al., 2011) and different vegetables (Abadias et al., 2008).

The high/low effectiveness of physical or chemical treatments on food decontamination are highly dependent on food surface properties such as hydrophobicity, electric charge and roughness, which may influence the adhesion and microbial distribution of food surfaces (Araújo et al., 2010). Additionally, hydrophobic/hydrophilic interactions between surfaces and bacteria are determinant in the process of adhesion/attachment and posterior inactivation of microbial cells through the various decontamination methods. These aspects certainly affect the difficulty of removing or inactivating microorganisms by chemical or physical agents and may explain the different levels of microbial reduction obtained by UV-C, AEW, NEW and SH in the diverse matrices. However, although the antimicrobial effect of UV-C illumination is dependent on the dose applied, food surface characteristics (roughness, hydrophobicity), initial bacterial inoculum, bacterial type and the low penetration capacity, it revealed to be more effective as a decontaminant of fresh-cut pear than the chemical sanitizers used (SH, AEW and NEW).

3.3. Effect of UV-C illumination and AEW and NEW on the quality parameters of fresh-cut pear

The effect of the UV-C and electrolyzed water (used in the antimicrobial studies described earlier) on the quality parameters of fresh-cut pear was studied before and after the application of the decontamination treatments. For this purpose, color, titratable acidity (TA), pH, soluble solid content (SSC) and firmness were measured on samples submitted to the different treatments and compared with the measurements of untreated samples immediately after cutting (AC) and 4 hours after cutting (CK). The results are shown in Table 1.

Table 1. 'Rocha' fresh-cut pear quality parameters ($L*$, H^0, SSC, TA, pH and Firmness) after treating with UV-C illumination (2.5, 5, 7.5 and 10 kJ/m^2) and after washing with acidic electrolyzed water (AEW), neutral electrolyzed water (NEW), sodium hypochlorite (SH) (100 mg/L of free chlorine), and with distilled water (DW). Untreated samples were used as control, right after cutting (AC) and 4 h after cutting (CK). For each value different letters (a, b, c, d) indicate signficant differences ($p < 0.05$) between treatments according to Duncan multiple range test.

Treatment	**Quality Parameter**					
	$L*$	H^0	**SSC**	**TA**	**pH**	**Firmness**
AC	96.2 (±0.57)a,b	-0.14 (±0.51)a	14.9 (±0.42)b	1.3 (±0.04)c	5.28 (±0.13)d	56.8 (±2.89)a
CK	96.4 (±1.18)a,b	0.93 (±0.54)a	14.3 (±0.15)b	0.94 (±0.04)a	5.15 (±0.18)b,c	65.8 (±4.97)a
UV2.5	96.93 (±1.10)a,b	1.45 (±0.10)a	13.7 (±0.75)b	0.98 (±0.08)a,b	4.5 (±0.13)a,b	61.6 (±7.62)a
UV5	95.88 (±1.61)a	1.52 (±0.02)a	13.13 (±1.18)b	1.14 (±0.04)a,b,c	5.06 (±0.13)b,c,d	60.87 (±3.74)a
UV7.5	98.59 (±1.03)b	1.41 (±0.08)a	14.5 (±1.2)b	1.16 (±0.02)a,b,c	4.80 (±0.24)a,b,c,d	64 (±5.62)a
UV10	97.54 (±0.92)b	1.46 (±0.03)a	12.37 (±0.64)a,b	1.09 (±0.08)a,b,c	4.85 (±0.24)a,b,c,d	67.17 (±3.74)a
AEW	97.94 (±0.94)b	0.42 (±0.99)a	11.9 (±0.95)a,b	1.34 (±0.15)c	4.65 (±0.31)a,b,c	54.93 (±8.85)a
NEW	93.59 (±1.17)a	0.46 (±0.95)a	14.47 (±1.05)b	1.14 (±0.10)a,b,c	4.40 (±0.07)a	55.93 (±6.53)a
SH	102.22 (±0.26)c	1.31 (±0.04)a	9.8 (±1.67)a	1.09 (±0.11)a,b,c	4.78 (±0.15)a,b,c,d	64.4 (±7.96)a
DW	96.96 (±1.22)a,b	0.90 (±0.59)a	14.83 (±0.23)b	1.23 (±0.04)b,c	4.62 (±0.03)a,b,c	55.87 (±3.43)a

The decontamination of pears using the different treatments did not induce changes in the parameter $L*$ ($p>0.05$) with the exception of pear treated with SH, where an increase in $L*$ was observed, meaning the color of the fruit surface became lighter after the SH washing ($p<0.05$). However, regarding Hue, no statistical differences were detected, among the samples ($p>0.05$). The value of the SSC (°Brix) was not affected by the decontamination treatments applied to the fresh-cut pears with the exception of the SH washing that caused a significant decrease in its value (from 14.3±0.15 in the CK to 9.8±1.67 in the SH washed pear) ($p<0.05$). This result may be explained by the fact that chlorine reacts with the organic material of the pear, resulting in a decreasing of some substances such as the sugars.

In regards to TA, a significant decrease of its value from 1.3 g malic acid/L pear juice to 0.94 g malic acid/L pear juice was observed when comparing the measurements performed in untreated pears immediately after cutting (AC) with the untreated pears analyzed 4 hours after cutting (CK) ($p<0.05$). However, there were no statistical differences among the CK and treated pears acidity values with the exception of AEW and DW washed pears. In the case of the pH, a decrease in its value was observed when comparing the measurements performed in untreated pears, immediately after cutting (AC), with the untreated pears analyzed 4 hours after cutting (CK) ($p<0.05$) (from 5.28±0.13 to 5.15±0.18). Except for pears washed with NEW, no differences were found among the pH value of fresh-cut pear treated when compared with the CK. Additionally, there were no significant differences in firmness among the different decontaminated treated fresh-cut pear ($p>0.05$). Several studies reported that UV-C illumination did not affect the quality parameters of fresh-cut fruits. In a study conducted by Graça et al. (2013) on fresh-cut apples submitted to UV-C illumination was observed that color, SSC and acidity were not significantly different after the treatment. Manzocco et al. (2009a, 2009b) also did not observe significant differences in color and firmness of fresh-cut melons and fresh cut apples treated with UV-C illumination, respectively. Regarding electrolyzed water, data obtained by Jia et al. (2015) with Chinese yam indicate that these chemical decontaminants may have a protecting effect on the color of the yam.

4. Conclusion

Minimally processed ‘Rocha’ pear (pH 5.28 and titratable acidity 1.3 g malic acid/L) has shown to be a good substrate for the survival and growth of *E. coli*, *S. enterica and Listeria* spp. The populations of *E. coli*, *S. enterica* and *Listeria* spp. were significantly reduced in fresh-cut pear after the application of UV-C and EW decontamination technologies. The use of UV-C resulted in microbial reductions higher than 2 log cfu/g while AEW, NEW and SH resulted in reductions of approximately 1 log cfu/g. In general, the UV-C dose of 7.5 kJ/m^2 caused the highest microbial reduction. UV-C and EW seem to be promising decontamination technologies as they allow the reduction of foodborne bacteria population and the amount of SH without greatly affecting the quality of fresh-cut pear. However, alone, none of them completely eliminate the pathogenic bacteria thus alerting the necessity for a strategy that combines different technologies in order to increase the safety of fresh-cut fruit. The results highlight the importance of preventing contamination and cross contamination, selecting an adequate decontamination technology and of maintaining a strict temperature control from production and processing until consumption of fresh-cut pear.

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Capítulo VI

Considerações finais e perspetivas futuras

Considerações finais

Tendo em conta o potencial da fruta minimamente processada (FMP) como veículo de microrganismos patogénicos, um dos objetivos do presente trabalho foi estudar a qualidade microbiológica desta fruta, comercializada em Portugal na região do Algarve. Este estudo permitiu concluir que a maçã fresca cortada continha os níveis médios mais altos das várias populações microbianas estudadas [microbiota aeróbia mesófila (7,7 log ufc/g) e psicrotrófica (7,6 log ufc/g), bactérias ácido-lácticas (BAL) (7,4 log ufc/g), bactérias coliformes (6,6 log ufc/g) e fungos filamentosos e leveduras (6,2 log ufc/g). As amostras de melancia minimamente processada revelaram também valores elevados de microrganismos mesófilos (6,4 log ufc/g), BAL (5,9 log ufc/g) e coliformes (5,2 log ufc/g). Em relação às contagens de microrganismos psicrotróficos, o melão Cantaloupe cortado apresentou as contagens médias (6,2 log ufc/g) mais altas a seguir à maçã e os morangos minimamente processados continham os valores médios mais altos de fungos (5,2 log ufc/g). O abacaxi e os morangos apresentaram os níveis médios mais baixos de bactérias coliformes (2,4 e 3,7 log ufc/g, respectivamente) e observou-se que todas as amostras de morangos estudadas apresentaram contagens deste grupo de enterobactérias inferiores a 10^5 ufc/g. Apesar dos valores dos parâmetros de qualidade microbiológica referidos anteriormente serem elevados, não foram detetados os microrganismos patogénicos de origem alimentar pesquisados (*E. coli*, *S. enterica*, *L. monocytogenes* e *C. sakazakii*). No caso da maçã, também não foram identificadas leveduras patogénicas. Contudo, a presença de coliformes em quase todas as amostras de fruta estudadas e de *S. aureus* em algumas (maçã) sugere o potencial deste tipo de alimentos como veículo de transmissão de microrganismos patogénicos e realça a importância de melhorar as boas práticas de fabrico e as medidas de controlo e de higiene em toda a cadeia de produção de fruta minimamente processada.

Em relação ao estudo dos principais grupos de microrganismos presentes quando as amostras mostravam sinais de degradação na fruta após a data limite de consumo ter terminado, constatou-se que no caso das amostras de maçã, os fungos, em particular as leveduras, apresentaram contagens significativamente mais elevadas, quando comparadas com as amostras estudadas durante o seu período de vida útil. As amostras de abacaxi cortado estudadas após o prazo de validade, continham níveis de microrganismos aeróbios mesófilos, psicrotróficos e fungos significativamente superiores àquelas estudadas antes desse limite ter sido ultrapassado. Nos restantes frutos (manga, papaia, melão, melão Gália, melão Cantaloupe e melancia), para além dos microrganismos aeróbios totais, psicrotróficos e dos fungos, observou-se que as BAL também se encontravam presentes em níveis significativamente mais elevados, quando a data limite de consumo da fruta tinha sido ultrapassada. Nas amostras de fruta em que houve um aumento significativo das contagens de BAL após o prazo de validade ter expirado, houve também uma diminuição significativa dos valores de pH da fruta (manga, papaia, melão, melão Gália, melão Cantaloupe e melancia). Na maçã e no abacaxi, os valores de pH estudados antes e depois do prazo de validade ter terminado não apresentam diferenças significativas. Em nenhuma das frutas estudadas, as contagens de bactérias coliformes determinadas durante o período de vida útil, antes do prazo de validade ter terminado, foram significativamente diferentes dos valores enumerados quando as frutas apresentavam sinais de degradação após aquele limite ter sido ultrapassado.

Na maçã fresca minimamente processada foi identificada uma grande variedade de leveduras sendo as espécies mais frequentemente isoladas, *Candida sake* (47,8 %), *Pichia fermentans* (25,4 %), *Hanseniaspora uvarum* (5,5 %) e *Candida incommunis* (5,5 %) seguidas das espécies *Meyerozyma guilliermondii* (2,5 %) e *Metschnikowia pulcherrima* (2,5 %). Foram também recolhidos vários isolados do género *Cryptococcus*, *Saturnispora* e da espécie psicrotrófica *Cystofilobasidium infirmominiatum*, os quais foram isoladas em frequências que variaram entre 1,7 e 1,3 %.

Apesar dos níveis dos parâmetros microbiológicos quantificados terem sido altos, a fruta minimamente processada analisada cumpriu os critérios microbiológicos de higiene e segurança alimentar de acordo com os regulamentos da Comissão Europeia (Regulamento (CE) Nº 2073/2005, Regulamento (CE) Nº 1441/2007).

O crescimento de microrganismos patogénicos nos alimentos durante o processamento, distribuição e/ou armazenamento parece ser determinante para ocorrência da maioria dos surtos de doenças causadas pela ingestão de alimentos (Codex Alimentarius Commission, 1999) e vários estudos têm demonstrado a capacidade de bactérias patogénicas para sobreviver e/ou crescer em frutas minimamente processadas armazenadas a diferentes temperaturas (Abadias *et al*, 2012;. Alegre *et al*, 2010a, 2010b; Dingman, 2000;. Lourenço *et al*, 2012). Além disso, os diferentes alimentos, incluindo os frutos, diferem na capacidade de suportar o crescimento de microrganismos. Por estas razões, estabeleceu-se como objetivo deste trabalho estudar a sobrevivência e o crescimento de bactérias patogénicas (*E. coli*, *S. enterica* e *Listeria* spp.) em pera 'Rocha' minimamente processada, a diferentes temperaturas. Os resultados permitiram concluir que estes microrganismos têm capacidade de sobreviver e/ou crescer na fruta cortada dependendo da temperatura e da extensão do período de armazenamento. Às temperaturas de 20 e 12 °C observou-se um crescimento exponencial das três bactérias nas primeiras 24 horas, tendo-se seguido uma fase estacionária durante o período em que decorreu o ensaio (8 dias). No final obtiveram-se populações das 3 bactérias que variaram entre 8,06±0,070 e 8,56±0,023 log ufc/g de pera minimamente processada. Nas amostras armazenadas a 8 °C, *E. coli* e *S. enterica* cresceram exponencialmente durante aproximadamente 3 dias, aumentando a sua população em mais de um ciclo logarítmico, entrando depois na fase estacionária. A 4 °C observou-se um ligeiro declínio de *E. coli,* durante os 8 dias que correspondeu a uma taxa de morte de 0,35±0,130 dia^{-1}. *S. enterica* manteve-se em fase de adaptação até ao oitavo dia.

No que diz respeito a *Listeria* sp., calcularam-se fases de adaptação inferiores a 24 h, 0,58±0,279 e 0,62±0,462 dia, seguidas de crescimento exponencial com taxas específicas de crescimento de 0,89±0,113 e 0,38±0,057 dia^{-1}, nas amostras armazenadas a 8 e 4 °C, respetivamente.

Os resultados apresentados permitem concluir que pera 'Rocha' fresca minimamente processada é um bom substrato para os três agentes patogénicos sobreviverem e se multiplicarem às temperaturas de incubação estudadas, incluindo as temperaturas de refrigeração. A 4 ° C, *Listeria* spp. foi capaz de crescer após uma fase de adaptação de cerca de 15 horas e *E. coli* e *S. enterica* após cerca de 8 dias. Estes resultados estão certamente relacionados com os fatores intrínsecos da pera utilizada nos estudos que possuía valores de pH de 5,28 (superiores aos de outras frutas) e acidez titulável de 1,3 g de ácido málico/L (inferiores aos de outras espécies) (pêssegos, pH 4,1 e acidez titulável 8,9 g de ácido málico/L; maçãs, pH 2.16 e acidez titulável 8,2 ácido málico g/L).

A temperatura de armazenamento é um dos principais fatores extrínsecos que determinam o crescimento microbiano nas matrizes alimentares. *L. monocytogenes* é um microrganismo psicrotrófico e a temperaturas de refrigeração induz um mecanismo de adaptação (resposta de choque ao frio) que lhe permite ajustar-se rapidamente e multiplicar-se, atingindo populações microbianas suficientes para causar doença, mesmo durante o período de vida útil dos alimentos prontos a ingerir, tais como a pera minimamente processada.

Por outro lado, a exposição a condições ligeiramente ácidas pode induzir mecanismos de tolerância, tais como a resposta de tolerância ao ácido, descrita em *S. enterica* e *E. coli* (Foster, 2001) e *L. monocytogenes* (Melo *et al*., 2015). Estes mecanismos, entre outros, permitem às bactérias sobreviver em produtos alimentares com um pH inferior ao seu pH ótimo, tais como frutos, e protegê-los, posteriormente, de condições de abaixamento de pH e/ou acidez mais graves. Mecanismos de adaptação como estes irão permitir a sobrevivência e o crescimento de microrganismos de origem alimentar, tendo implicações gravosas na segurança dos alimentos, tais como alimentos ácidos armazenados a baixas temperaturas.

Os resultados obtidos alertam para o risco de ocorrência de multiplicação de bactérias patogénicas em pera fresca minimamente processada a temperaturas de 4 °C, durante o período de vida útil do alimento. Na presença de contaminações, em condições de armazenamento favoráveis à sobrevivência e desenvolvimento de mecanismos de adaptação e crescimento, podem atingir-se níveis populacionais não conformes com os critérios microbiológicos da União Europeia (*Salmonella* sp. – tolerância zero; *L. monocytogenes* – 100 ufc/g em alimentos prontos a comer) passíveis de causarem doença em humanos.

Estes resultados alertam para a necessidade de implementação de métodos de desinfeção/descontaminação eficazes. Desta forma, outro objetivo do presente trabalho foi avaliar a capacidade de desinfeção de um método físico, a iluminação ultravioleta-C (UV-C), em pera 'Rocha' e em maçã 'Golden' e de um método químico, a água electrolisada (AE) (ácida e neutra), em pera ‘Rocha’. Em ambos os casos, a pera e a maça minimamente processadas foram previamente inoculadas com suspensões de *E. coli*, *S. enterica* e *Listeria* spp. Os resultados obtidos foram sempre comparados com a desinfeção realizada com soluções de hipoclorito de sódio, método utilizado normalmente na indústria de produção de alimentos minimamente processados.

No que diz respeito à pera minimamente processada, os resultados obtidos permitiram afirmar que as populações de *E. coli*, *S. enterica* e *Listeria* spp. foram reduzidas significativamente após a aplicação de tratamentos de desinfeção utilizando a iluminação UV-C e a AE. A dose UV7.5 kJ/m^2 revelou-se uma estratégia de descontaminação UV-C mais eficiente, tendo a sua aplicação resultado em reduções dos três agentes patogénicos de origem alimentar superiores a 2,4 log ufc/g, quando inoculados em cultura pura ou cultura mista (constituída por uma suspensão das três bactérias). As desinfeções levadas a cabo recorrendo a UV-C foram mais eficazes do que os realizados com a solução de hipoclorito de sódio (100 ppm) que resultaram em reduções inferiores a 1 log ufc/g, com exceção de *E. coli* e *Listeria* sp. (quando inoculados em culturas de uma única). Em relação às desinfeções levadas a cabo com a AE, o nível de reduções microbianas alcançado não ultrapassou 1,1 log ufc/g. As desinfeções com AE resultaram em reduções que não foram significativamente diferentes das obtidas com soluções de hipoclorito de sódio. Pode concluir-se que as desinfeções realizadas utilizando os métodos químicos resultaram em reduções microbianas mais baixos do que as obtidas quando a iluminação UV-C foi aplicada como método de desinfeção.

A eficácia da iluminação UV-C na inativação de *E. coli*, *S. enterica* e *L. innocua* foi também estudada *in vitro*, em amostras de maçã minimamente processada. Neste estudo a dose de 1,0 kJ/m^2 inibiu totalmente o crescimento de *E. coli* e de *S. enterica* e permitiu obter uma redução da população de *L. innocua* acima de 7,5 log ufc/g. A dose de 1,0 kJ/m^2 apresentou-se como uma alternativa à lavagem de maçã minimamente processada com soluções de hipoclorito de sódio (100 ppm de cloro livre) uma vez que permitiu obter contagens dos microrganismos mais baixas em todos os casos estudados.

Nas amostras inoculadas com uma cultura isolada de cada um dos microrganismos e iluminadas com esta dose de UV-C (1,0 kJ/m^2), foram observadas reduções entre 1,6 e 1,1 log ufc/g após o tratamento e entre 1,7 e 0,5 log ufc/g após 15 dias de armazenamento a 4 °C. Em relação às amostras inoculadas com uma mistura dos três microrganismos, foram observadas reduções entre 2,0 e 4,1 log ufc/g no início do período de armazenamento (tempo 0) e de cerca de 1,6 log ufc/g após 15 dias de armazenamento a 4 °C. As lavagens com a solução de hipoclorito de sódio a (100 ppm) não mostraram diferenças significativas dos tratamentos com a iluminação UV-C na dose 0,5 kJ/m^2 na redução dos microrganismos.

A iluminação UV-C e a AE apresentaram-se como métodos de desinfeção promissores para a desinfeção de fruta minimamente processada, pois permitiram reduções da população de bactérias patogénicas de origem alimentar iguais ou superiores às reduções obtidas aquando da aplicação de soluções de hipoclorito de sódio (100 ppm), sem afetar significativamente os parâmetros de qualidade (cor, pH, acidez titulável e °Brix) das maçãs e peras minimamente processadas. No entanto, a iluminação UV-C, permitiu, na maioria dos casos, obter reduções das populações de microrganismos superiores às obtidas com a lavagem a água electrolisada e com a solução de hipoclorito de sódio sem afetar a qualidade da fruta. Nenhum dos métodos utilizados conseguiu eliminar completamente as bactérias patogénicas alertando, assim, para a necessidade de uma estratégia que combine diferentes tecnologias, de forma a aumentar a segurança microbiológica da fruta cortada.

Os resultados obtidos destacam a importância de prevenir as contaminações microbianas, de selecionar tecnologia(s) de descontaminação adequadas e eficientes e de manter um rigoroso controlo de temperatura durante a produção, processamento, distribuição e comercialização de fruta minimamente processada.

Perspetivas futuras

A iluminação UV-C e a água electrolisada (ácida e neutra), enquanto métodos de desinfeção de fruta minimamente processada mostraram-se eficazes na redução das populações das bactérias *E. coli, S. enterica* e *Listeria* sp.. Contudo, os trabalhos realizados poderiam ser complementados com outros estudos tais como:

- Testar a eficácia da iluminação UV-C e da lavagem com água electrolisada ácida e neutra no controlo dos microrganismos responsáveis pela degradação, nomeadamente microrganismos aeróbios mesófilos, microrganismos aeróbios psicrotróficos, bactérias ácido-lácticas e leveduras;
- Realizar ensaios de avaliação das características sensoriais da fruta, recorrendo a um painel de provadores, após aplicação dos tratamentos de desinfeção utilizando UV-C e água electrolisada, nomeadamente nas condições que se revelaram mais eficazes no decorrer do presente trabalho;
- Testar a aplicação combinada dos dois métodos (UV-C e água electrolisada) de modo a obter uma maior eficácia de desinfeção/descontaminação;
- Proceder à aplicação das condições de desinfeção que se revelaram mais eficazes a um nível semi-industrial e industrial (fazer o *scale-up* dos métodos de desinfeção estudados);
- Testar a iluminação UV-C e a água electrolisada noutras frutas minimamente processadas (manga, melão, entre outras);

Para além da iluminação UV-C e da água electrolisada pretende-se estudar a aplicação de outras técnicas de desinfeção em fruta minimamente processada, que têm revelado resultados promissores, tais como a luz pulsada e o ozono.
Uma outra área de trabalho interessante é o estudo do efeito de algumas bactérias ácido-lácticas, isoladas no decurso do presente trabalho (resultados não apresentados), enquanto agentes de biocontrolo do crescimento de bactérias patogénicas em fruta minimamente processada. Na sequência deste estudo, podere-se-ia-a avaliar a possibilidade de desenvolver fruta minimamente processada probiótica.

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