UNIVERSIDAD DE LLEIDA

Escuela Técnica Superior de Ingeniería Agraria

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USO DE SUSTANCIAS ANTIMICROBIANAS NATURALES EN COMBINACIÓN CON COMPUESTOS ESTABILIZADORES DE LA CALIDAD PARA CONTROLAR MICROORGANISMOS PATÓGENOS Y EXTENDER LA VIDA ÚTIL DE FRUTAS FRESCAS CORTADAS

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Tesis Doctoral

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RESUMEN

El consumo de frutas frescas cortadas y zumos de frutas no pasteurizados ha incrementado significativamente en los últimos años debido a la creciente demanda de productos sanos con bajo contenido calórico y características similares a las del producto fresco. No obstante, estos productos pueden contaminarse con microorganismos patógenos o deteriorativos por una inapropiada manipulación o almacenamiento, dando lugar a riesgos de enfermedades microbianas y deterioro del producto. De hecho, el número de brotes y casos de enfermedades causadas por consumo de esos productos ha aumentado notablemente, razón por la cual el uso de compuestos naturales que garanticen la inocuidad y calidad de las frutas frescas cortadas y zumos de frutas no pasteurizados ha aumentado. El objetivo principal de esta investigación fue evaluar la efectividad de diferentes antimicrobianos para controlar microorganismos patógenos tales como *Listeria monocytogenes*, *Salmonella* Enteritidis y *Escherichia coli* O157:H7 y extender la vida útil de frutas frescas cortadas. Primeramente se determinaron las concentraciones mínimas inhibitorias (CMI) y bactericidas (CMB) de ácido málico y diferentes aceites esenciales (EOs) y sus compuestos activos en zumos de manzana, pera y melón. Después, esas sustancias fueron aplicadas en combinación con compuestos estabilizadores de la calidad directamente por inmersión o indirectamente a través de recubrimientos comestibles a base de alginato a manzanas, peras y melones frescos cortados para garantizar su inocuidad y calidad. Los zumos de manzana, pera y melón fueron inhibitorios para *L. monocytogenes*, *S.* Enteritidis y *E. coli* O157:H7 a 5ºC, sin embargo a 20 y 35ºC, se necesitaron concentraciones mínimas de ácido málico de 0,2% para *L. monocytogenes* y *S*. Enteritidis y 0,4% para *E. coli* O157:H7 para inhibir su crecimiento en zumo de melón. En general, concentraciones de ácido málico de 2% en zumos de manzana y pera y 2,5% en zumo de melón fueron requeridas para reducir las poblaciones de *L. monocytogenes*, *S.* Enteritidis y *E. coli* O157:H7 en más de 5 ciclos log a 5ºC, mientras a 20 y 35ºC concentraciones más bajas fueron suficientes para alcanzar ese efecto. Por otra parte, los aceites esenciales de canela, clavo, hierba de limón y palmarosa así como también sus compuestos activos mostraron actividad antimicrobiana sobre los microorganismos patogénicos pero en diferentes grados. Así la efectividad de esas sustancias dependió del tipo de microorganismo, tipo de zumo y concentración de la sustancia. Una combinación de ácido málico al 2,5%, N-acetíl-L-cisteína al 1% glutatione al 1% y lactato de calcio al 1% aplicada por inmersión redujo las poblaciones de *L. monocytogenes* y *S.* Enteritidis inoculadas en manzanas y peras frescas cortadas en más de 5 ciclos log el mismo día de la preparación de las muestras (t = 0 días), mientras que la población de *E. coli* O157:H7 mostró una mayor ácido-resistencia, alcanzando esa reducción a partir de los 3 y 14 días en esas frutas respectivamente. Además esa combinación de sustancias causó una disminución en la tasa de crecimiento y una prolongación de la fase lag de las poblaciones de aerobios mesófilos, psicrófilos y mohos y levaduras, extendiendo la vida útil desde un punto de vista microbiológico hasta 23 días en piezas de manzanas y por más de 30 días en piezas de peras. Así mismo, un menor consumo de O_2 y una menor producción de

CO_2, etileno y etanol, así como también un mejor mantenimiento de la textura y el color fueron logradas con el uso de esa combinación de sustancias, alcanzándose así una extensión de la vida útil fisicoquímica. Por otra parte la incorporación de ácido málico junto con compuestos estabilizadores de la calidad en recubrimientos comestibles a base de alginato fue efectiva para reducir las poblaciones de *E. coli* O157:H7 y *S.* Enteritidis inoculadas en piezas de manzana y melón así como también para inhibir el crecimiento de la flora nativa, sin embargo un mayor efecto sobre esos microorganismos fue logrado cuando ambos ácido málico y EOs o sus principales compuestos activos fueron incorporados en los recubrimientos, mostrando ser el aceite esencial de hierba de limón la sustancia antimicrobiana más efectiva. Este efecto antimicrobiano fue además potenciado con el aumento de las concentraciones de EOs. Una extensión de la vida útil microbiológica de piezas de manzana y melón también fue alcanzada con la incorporación de estas sustancias, sin embargo algunas características fisicoquímicas y sensoriales de las frutas como textura, color, olor y sabor fueron afectadas. Los resultados demostraron que el uso de antimicrobianos naturales por inmersión o a través de su incorporación en recubrimientos comestibles a base de alginato es una buena alternativa para garantizar la inocuidad y calidad de frutas frescas cortadas.

RESUM

El consum de fruites fresques tallades i sucs de fruites no pasteuritzats s'ha incrementat significativament en els darrers anys degut a la creixent demanda de productes sans de baix contingut calòric i característiques similars a les del producte fresc. Tot i això, aquests productes poden contaminar-se amb microorganismes patògens o deterioratius per una inadequada manipulació o emmagatzematge, donant lloc a riscs de malalties microbianes i deteriorament del producte. De fet, el nombre de brots i casos de malalties causades pel consum d'aquests productes ha augmentat notablement, motiu pel qual l'ús de composts naturals que garanteixin la seguretat i qualitat de les fruites fresques tallades i sucs de fruites no pasteuritzats ha augmentat. L'objectiu principal d'aquesta recerca ha estat avaluar l'efectivitat de diferents antimicrobians per a controlar microorganismes patògens tals com *Listeria monocytogenes*, *Salmonella* Enteritidis i *Escherichia coli* O157:H7 i allargar la vida útil de fruites fresques tallades. Inicialment les concentracions mínimes inhibitòries (CMI) i bactericides (CMB) d'àcid màlic i diferents olis essencials (EOs) i els seus compostos actius van ser determinats en sucs de poma, pera i meló. Després aquestes substàncies han estat aplicades en combinació amb composts estabilitzadors de la qualitat directament per immersió o indirectament a través de recobriments comestibles a base d'alginat a pomes, peres i melons frescs tallats per a garantir la seva seguretat i qualitat. Els sucs de poma, pera i meló van ser inhibitoris per a *L. monocytogenes*, *S.* Enteritidis i *E. coli* O157:H7 a 5ºC, no obstant a 20 i 35ºC concentracions mínimes d'àcid màlic de 0,2% per a *L. monocytogenes* i *S.* Enteritidis i 0,4% per a *E. coli* O157:H7 van ser requerides per a inhibir els seus creixements en suc de meló. En general, concentracions d'àcid màlic de 2% en sucs de poma i pera i de 2,5% en suc de meló van ser requerits per a reduir les poblacions de *L. monocytogenes*, *S.* Enteritidis i *E. coli* O157:H7 en més de 5 cicles log a 5ºC, mentre a 20 i 35ºC concentracions més baixes van ser suficients per a aconseguir aquest efecte. D'altra banda, els olis essencials de canyella, clan, herba de llimona i palmarosa així com també els seus compostos actius van mostrar activitat antimicrobiana sobre els microorganismes patogènics però en diferents graus. Així l'efectivitat d'aquestes substàncies va dependre del tipus de microorganismes, tipus de suc i concentració de la substància. Una combinació d'àcid màlic al 2,5%, N-acetil-L-cisteina a l'1% glutatione a l'1% i lactat de calci a l'1% aplicada per immersió va reduir les poblacions de *L. monocytogenes* i *S.* Enteritidis inoculades en pomes i peres fresques tallades en més de 5 cicles log el mateix dia de la preparació de les mostres (t = 0 dies), mentre que la població de *E. coli* O157:H7 va mostrar una major àcid-resistència, aconseguint aquesta reducció a partir dels 3 i 14 dies en aquestes fruites respectivament. A més a més, aquesta combinació de substàncies va originar una disminució en la tasa de creixement i una prolongació de la fase lag de les poblacions d'aerobis mesòfils, psicròfils i floridures i llevadures, allargant la vida útil des d'un punt de vista microbiològic fins a 23 dies en peces de poma i per més de 30 dies en peces de pera. Així mateix, un menor consum d'O_2 i una menor producció de CO_2, etilé i etanol, com també un millor manteniment de la textura i el color foren aconseguits amb l'ús d'aquesta combinació de substàncies, aconseguint

així un allargament de la vida útil fisicoquímica. D'altra banda, la incorporació d'àcid màlic junt amb composts estabilitzadors de la qualitat en recobriment comestibles a base d'alginat va ser efectiva per a reduir les poblacions d'*E. coli* O157:H7 i *S*. Enteritidis inoculades en peces de poma i meló així com també per a inhibir el creixement de la flora nativa, tot i això un major efecte sobre aquells microorganismes es va aconseguir quan ambdós àcids màlic i EOs o els seus principals composts actius van ser incorporats en els recobriments, mostrant ser l'oli essencial d'herba de llimona la substància antimicrobiana més efectiva. Aquest efecte antimicrobià fou a més a més potenciat amb l'augment de les concentracions d'EOs. Una extensió de la vida útil microbiològica de peces de poma i meló fou també aconseguida amb la incorporació d'aquestes substàncies, no obstant algunes característiques fisicoquímiques i sensorials de les fruites com textura, color, olor i gust van ser afectades. Els resultats demostraven que l'ús d'antimicrobians naturals per immersió o a través de la seva incorporació en recobriments comestibles a base d'alginat és una bona alternativa per a garantir la seguretat i qualitat de fruites fresques tallades.

SUMMARY

The consumption of fresh-cut fruits and unpasteurized juices has substantially risen over the last years mostly due to the increasing demand of healthy food with low caloric contents and fresh-like characteristics. Nonetheless, those products can contaminate with pathogenic and deteriorative microorganisms as a consequence of inappropriate manipulation and storage conditions, resulting in risks of microbial diseases and spoilage of the product. In fact, the number of outbreaks and cases of illness caused by consumption of fresh-cut fruits and unpasteurized juices has notably increased reason by which, the use of natural compounds that assure the safety and quality of fresh-cut fruits and unpasteurized fruit juices has increased. To evaluate the effectiveness of different antimicrobials to control pathogenic microorganisms such as *Listeria monocytogenes*, *Salmonella* Enteritidis and *Escherichia coli* O157:H7 and extend the shelf-life of fresh-cut fruits was the main objective of this research. Minimal inhibitory (MIC) and bactericidal concentrations (MBC) of malic acid and essential oils (EOs) and their active compounds against those pathogens were firstly determined in apple, pear and melon juices. Then, those substances were applied in combination with quality stabilizing compounds directly by dipping treatments or indirectly through the alginate-based edible coatings to fresh-cut apple, pear and melon to assure their safety and quality. Apple, pear and melon juices were inhibitory for *L. monocytogenes*, *S.* Enteritidis and *E. coli* O157:H7 at 5ºC, however at 20 and 35ºC minimal concentrations of 0.2% of malic acid for *L. monocytogenes* and *S.* Enteritidis and 0.4% for *E. coli* O157:H7 were required to inhibit their growth in melon juice. In general, concentrations of malic acid of 2% in apple and pear juices and 2.5% in melon juice were necessary to reduce more than 5 log cycles of *L. monocytogenes*, *S.* Enteritidis and *E. coli* O157:H7 at 5ºC, whereas at 20 and 35ºC lower concentrations were enough to reach that effect. On the other hand, EOs of cinnamon, clove, lemongrass and palmarosa as well as their active compounds showed antimicrobial activity over pathogenic microorganisms but in different grade. Thus, the effectiveness of those substances depended on the microorganism type, kind of juice and concentration of the substance, thus resulting, the essential oil of lemongrass the substance more effective to inactivate those microorganisms. A combination of 2.5% D-L malic acid, 1% N-acetyl-L-cysteine, 1% glutathione and 1% calcium lactate applied through dipping treatment reduced the populations of *L. monocytogenes* and *S.* Enteritidis inoculated in fresh-cut apples and pears in more than 5 log cycles the same day of sample preparation (t = 0 days), whereas the population of *E. coli* O157:H7 showed a higher acid-resistance, reaching that reduction from 3 and 14 days in those fruits respectively. In addition, that combination provoked a decrease of the growth rate and prolonged the lag phase of mesophilic aerobic, psychrophilic and yeast and molds populations, extending the shelf-life from a point of view microbiological until 23 days in apple pieces and by more than 30 days in fresh-cut pears. Likewise, a lower consumption of O_2 and a lower production of CO_2, ethylene and ethanol, as well as a better maintenance of

the firmness and color were achieved with the use of that substances combination, thus reaching a physicochemical shelf-life extension. On the other hand, the incorporation of malic acid with quality stabilizing compounds into alginate-based edible coatings was effective to reduce the populations of *E.coli* O157:H7 and *S.* Enteritidis inoculated in apple and melon pieces as well as to inhibit the native flora growth. However, a higher effect over those microorganisms was achieved when both malic acid and EOs or their main active compounds were added into coatings, showing the essential oil of lemongrass to be the most effective antimicrobial substance. That antimicrobial effect was also intensified with the increase of the EOs concentrations. An extension of the microbiological shelf-life of apple and melon pieces was also reached with the incorporation of those substances. Nonetheless, some physicochemical and sensory characteristics of the fruits such as firmness, color, flavour and taste were affected. The results demonstrated that the use of natural antimicrobials by immersion or through their incorporation into alginate-based edible coatings is a good alternative to assure the safety and quality of the fresh-cut fruits.

INDICE

Use of natural antimicrobials to control pathogenic and spoilage microorganisms in fresh-cut fruits and fruit juices. A review

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ABSTRACT

Studies on the use of natural antimicrobials to prevent quality loss in fresh-cut fruits and unpasteurized juices while assuring microbiological safety have significantly increased in the last years. Consumers demand healthy, fresh-like and safe foods that contain low amounts of preservatives. In such sense, the effectiveness of antimicrobial agents from animal, plant and microbial origin, directly or indirectly added to fresh-cut fruits and derivatives, has been studied by different researchers. Antimicrobials from animal origin such as lactoperoxidase and lysozyme enzymes have shown to be effective in reducing the presence of *E. coli*, *Shigella* and *Salmonella* in apple, orange and tomato juices as well as in inhibiting natural flora in grape juice and apple cider, whereas chitosan was useful to inhibit the natural occurring microflora in fresh-cut papaya and mango or in apple juice. In the same way, antimicrobials from plants such as essential oils, herbs and spices have been effective in reducing spoilage flora in fresh-cut fruits and pathogenic microorganisms in juices. Furthermore, aldehydes and organic acids can reduce pathogenic microorganisms populations and also effectively inhibit the native flora of fresh-cut fruits, whereas, esters show less efficiency against pathogens. Eventually, antimicrobials from microorganisms such as nisin have been used in fresh-cut fruit and juices to delay the proliferation of naturally occurring microbial flora and to inhibit the growth of *E. coli* O157:H7 and *Salmonella* Typhimurium in fruit juices. Nevertheless, the use of these compounds at commercial level is still limited due to several factors such as their influence over the sensory characteristics or, in some cases, regulatory issues concerning their use.

1- INTRODUCTION

Consumption of ready-to-eat fresh-cut fruits and juices has substantially risen over the last years (Corbo et al., 2000; Diaz-Cinco et al., 2005; Rico et al., 2007), mostly due to the increasing demand for low caloric food products with fresh-like characteristics. In addition, there is scientific evidence that consumption of fruits and vegetables helps to prevent many degenerative diseases such as cardiovascular problems and several cancers (Rico et al., 2007). However, as a consequence of inappropriate manipulation and storage conditions, both pathogenic and/or deteriorative microorganisms may contaminate the product, thus increasing the risk of microbial diseases and spoilage of the product (Beuchat, 1996; Diaz-Cinco et al., 2005). In fact, the number of outbreaks and cases of illness caused by consumption of fresh-cut fruits and unpasteurized juices has increased in the last years (Harris et al., 2003).

Quality losses in fresh-cut fruits and unpasteurized juices may occur as a consequence of microbiological, enzymatic, chemical, or physical changes. Microbiological alterations are of great importance, since they may entail consumer hazards, especially when microbial toxins or pathogenic microorganisms are found in the product, in addition to economic losses as a result of microbial spoilage. Many food preservation strategies such as chilling, freezing, water activity reduction, nutrient restriction, acidification, modified atmosphere packaging, fermentation, non-thermal physical treatments, as well as the use of antimicrobials have been traditionally applied to control microbial growth (Davidson, 2001). However, the interest in the use of natural antimicrobials to prevent fresh-cut fruits and unpasteurized juices from microbiological spoilage while assuring safety and mantaing quality characteristics has significantly increased in the last years, due to the high demand of healthy, fresh-like and safe foods that contain as low amounts of preservatives as possible (Soliva-Fortuny and Martín-Belloso, 2003).

Antimicrobial agents are included within of the food additives group. Therefore, their use in foods is ruled both by international and national regulations. Hence, different countries have their own regulations with a list of approved additives (European Parliament and Council Directive Nº 95/2/EC, 1995; USFDA, 2006 and 2007). The Food and Drug Act (FDA), European Union standards, and Codex Alimentarius, which constitutes the FAO/WHO joint regulatory body, are the foremost governmental regulations concerning food additives (Raju and Bawa, 2006). According to those Regulatory Organizations, the antimicrobials

reported in this work are generally recognized as safe (GRAS); however, some limits based on the allowed daily intake (ADI) can be established by each Regulatory Organization.

This review presents a compilation of the different works studying the use of natural antimicrobials in fresh-cut fruits and juices to maintain their safety and quality.

2- MICROBIOLOGY OF FRESH-CUT FRUITS AND UNPASTEURIZED FRUIT JUICES

Foods of plant origin such as fruits and vegetables have heterogeneous characteristics in their compositions. Consequently, the microflora in these products may substantially differ depending on medium pH, nutrient availability and water activity, among other factors (Kalia and Gupta, 2006). Fruits may be contaminated with pathogenic and spoilage microorganisms either during their growing in fields, orchards, vineyards or greenhouses, or during harvesting, post-harvest handling, and distribution (Cherry, 1999; Beuchat, 2002). Fresh fruits have a natural protective barrier (skin) that acts effectively against most plant spoilage and pathogenic microorganisms; however, this protection is eliminated during processing, thus exposing the fruit flesh to unfavorable environmental conditions as well as to a possible contamination with pathogenic microorganisms including bacteria, viruses and parasites (Nguyen-the and Carlin, 1994; Brackett, 1994). Hence, the number of documented outbreaks of human infections associated with consumption of fresh-cut fruits and unpasteurized fruit juices has increased in recent years (Table 1 and 2).

Few surveys of pathogens in fresh-cut fruits and fruit juices have been carried out and reported in the literature. In this way, Harris et al., (2003) reported absence of *Salmonella* in a total of 336 samples of freshly peeled oranges or tangerines. Likewise, Martínez et al., (2000) reported absence of *L. monocytogenes* in minimally processed fruit salads including papaya, apple, watermelon, grape, guava, and pineapple. On the other hand, Sado et al., (1998) found that 2 samples of a total of 50 analyzed, corresponding to an apple juice and an apple/raspberry juice, were positive for *L. monocytogenes*. In contrast, survival and growth of pathogenic microorganisms in fresh-cut fruits and fruit juices has been more extensively studied. In such sense, challenge studies have been performed to evaluate the behavior of *Campylobacter jejuni*, *E. coli* O157:H7, *Salmonella* spp., *L. monocytogenes*, *Staphylococcus aureus* and *Shigella* in several products (Table 3 and 4).

Table 1. Outbreaks of foodborne illness caused by pathogenic bacteria associated with fresh fruits

Causal agent	Year	Fruits	Cases (death)	Place
E. coli O157:H7	2005	Fruit salad	18	Home
Salmonella ser. Braenderup	2005	Roma tomatoes	84	Restaurant or deli
Salmonella ser. Braenderup	2004	Roma tomatoes	137	Restaurant, home
Salmonella multiserotypes	2004	Roma tomatoes	429	
Salmonella spp.	2003	Strawberry	13	
Salmonella ser. Muenchen	2003	Cantaloupe, Honeydew melons	58	
Salmonella ser. Newport	2003	Honeydew melons	68	
Salmonella ser. Berta	2002	Watermelon	29	
Salmonella ser. Poona	2002	Cantaloupe melon	26	
Salmonella ser. Newport	2002	Tomatoes	510	
Salmonella ser. Newport	2002	Fruit salad	51	
Salmonella ser. Poona	2001	Honeydew melons, watermelon	23	Restaurant
Salmonella ser. Saintpaul	2001	Mango	26	Private home
Salmonella ser. Poona	2001	Watermelon	23	
Salmonella ser. Poona	2001	Cantaloupe, Honeydew melons	50	Private home
Salmonella ser. Senftenberg	2001	Green grapes	40	Private home
E. coli O157:H7	2001	Pear	14	School
E. coli O157:H7	2000	Watermelon	736	Restaurant
E. coli O157:H7	2000	Red grape	14	Grocery store
Salmonella ser. Poona	2000	Cantaloupe melon	46	
Salmonella ser. Thompson	2000	Tomato	43	Private home
Salmonella ser. Enteritidis	1999	Honey dew melons / watermelon	82	School
Salmonella ser. Newport	1999	Mango	79	Multiple
Salmonella ser. Baildon	1998	Tomatoes	>85(3)	Multiple
Salmonella ser. Oranienburg	1998	Mango	9	Private home
Salmonella ser. Oranienburg	1998	Cantaloupe	22	Various
E. coli O157:H7	1997	Melon	9	Private home
Salmonella ser. Saphra	1997	Cantaloupe melon	24	Restaurant, grocery store
E. coli O157:H7	1993	Cantaloupe	27	Restaurant
Salmonella ser. Montevideo	1993	Tomatoes	100	Restaurant
Salmonella ser. Poona	1991	Cantaloupe	>400	Multiple
Salmonella ser. Javiana	1990	Fresh tomatoes	176	Day care center, restaurant
Salmonella ser. Chester	1990	Cantaloupe	25,000(2)	Unknown
Salmonella ser. Miami	1954	Watermelon	17(1)	Supermarket

Adapted from Harris et al., 2003 and CDC, 2007

Table 2. Outbreaks of foodborne illness caused by pathogenic bacteria associated with fruit juices

Causal agent	Year	Fruit juice	Cases (death)	Place
Salmonella ser. Thiphymurium & Saintpaul	2005	Orange juice unpasteurized	157	Restaurant, deli, private home
Salmonella ser. Enteriditis	2000	Orange, grapefruit and lemonade juice	74	Multiple places
Salmonella ser. Muenchen	1999	Orange juice unpasteurized	398(1)	
Salmonella ser. Typhimurium	1999	Orange juice	427	Retail
Salmonella ser. Anatum	1999	Orange juice unpasteurized	10	Other
Salmonella ser. Typhimurium	1999	Mamey juice unpasteurized	13	
E. coli O157:H7	1999	Apple cider unpasteurized	5	Private home
E. coli O157:H7	1998	Apple juice	14	Farm, Home
E. coli O157:H7	1997	Apple cider unpasteurized	6	Farm
E. coli O157:H7	1996	Apple cider unpasteurized	56	Multiple
E. coli O157:H7	1996	Apple juice unpasteurized	71(1); 14HUS	Community
E. coli O157:H7	1996	Apple cider unpasteurized	14 (3)	Small cider mill
E. coli O157:H7	1996	Apple cider unpasteurized	6	Small cider mill
Salmonella ser. Hartford, Gaminara & Rubislaw	1995	Orange juice	62	Theme park
E. coli O157:H7	1992	Orange juice	6	Roadside vendor
E. coli O157:H7	1991	Apple cider	23; 4HUS	Community
Salmonella ser. Javiana	1991	Watermelon juice	39	Indoor picnic, school party
Salmonella ser. Enteriditis	1991	Orange juice	600	
E. coli O157:H7	1980	Apple juice unpasteurized	14(1); 14HUS	Local market
Salmonella ser. Typhimurium	1974	Apple cider	296	Farm and small retail outlets

HUS: people with hemolytic uremic syndrome.
Adapted from Powell and Luedtke, 2000; Harris et al., 2003 and CDC, 2007

Table 3. Survival and growth of pathogenic bacteria in fresh-cut fruits

Pathogen	Fresh-cut fruit	Fruit pH	Temperature (°C)	Storage time (hours)	Initial/final counts (Log CFU/g)	Reference
Campylobacter jejuni	Watermelon	3.0	25 - 29	6	2.9/2.0	Castillo and Escartin, 1994
Campylobacter jejuni	Watermelon	5.5	25 - 29	6	2.7/2.1	Castillo and Escartin, 1994
Campylobacter jejuni	Papaya	3.0	25 - 29	6	3.3/Nd	Castillo and Escartin, 1994
Campylobacter jejuni	Papaya	5.0	25 - 29	6	2.8/1.7	Castillo and Escartin, 1994
Escherichia coli O157:H7	Melon (Cantaloupe)	7.01	25 and 5	34 and 34	3.0/7.0 and 3.1/3.1	Del Rosario and Beuchat, 1995
Escherichia coli O157:H7	Watermelon	5.56	25 and 5	34 and 34	3.0/8.7 and 3.0/3.0	Del Rosario and Beuchat, 1995
Escherichia coli O157:H7	Apples (Red delicious)	4.10	4, 10 and 25	432, 288 and 120	7.5/6.8, 7.5/5.8 and 7.5/8.5	Fisher and Golden, 1998
Escherichia coli O157:H7	Apples (Golden delicious)	3.84	4, 10 and 25	432, 288 and 120	7.5/7.2, 7.5/6.8 and7.5/8.2	Fisher and Golden, 1998
Escherichia coli O157:H7	Apples (Rome)	3.70	4, 10 and 25	432, 288 and 120	7.5/6.8, 7.5/7.0 and 7.5/7.5	Fisher and Golden, 1998
Escherichia coli O157:H7	Apples (Winesap)	3.47	4, 10 and 25	432, 288 and 120	7.5/7.0, 7.5/7.0 and 7.5/7.0	Fisher and Golden, 1998
Escherichia coli O157:H7	Oranges (Hamlin)	6.0-6.5	4, 8 and 24	336, 336 and 24	3.5/3.2, 3.5/2.5 and 3.5/7.5	Pao et al., 1998
Escherichia coli O157:H7	Apples (Golden delicious)	Not reported	24	48	2.0/5.5	Janisiewicz et al., 1999
Escherichia coli O157:H7	Pear (Cactus)	5.9	4, 8, 12 and 20	336	3.0/4.8, 3.0/5.1, 3.0/3.8 and 3.0/Nd	Corbo et al., 2005
Salmonella multiserotypes	Melon (Cantaloupe)	6,67	23 and 5	24 and 24	2.0/7.2, 2.0/1.6	Golden et al., 1993
Salmonella multiserotypes	Melon (Honeydew)	5.95	23 and 5	24 and 24	2.0/8.0 and 2.0/1.8	Golden et al., 1993
Salmonella multiserotypes	Watermelon	5.90	23 and 5	24 and 24	2.0/8.6 and 2.0/1.9	Golden et al., 1993
Salmonella ser. Typhi	Papaya	5.69	25 - 27	6	2.9/4.3	Fernandez-Escartin et al., 1989
Salmonella ser. Typhi	Papaya	3.59	25 - 27	6	3.0/3.8	Fernandez-Escartin et al., 1989
Salmonella spp.	Oranges (Hamlin)	6.0-6.5	4, 8 and 24	336, 336 and 24	4.4/3.5, 4.4/2.5 and 4.4/7.6	Pao et al., 1998
Salmonella ser. Chester	Apples (Golden delicious)	4.1	8	66	5.5/5.3	Liao and Sapers, 2000
Salmonella ser. Chester	Apples (Golden delicious)	4.1	20	66	5.5/10.4	Liao and Sapers, 2000
Salmonella ser. Enteritidis	Apple (Granny Smith)	4.1	20	150	4.5/7.7	Lanciotti et al., 2003
Listeria monocytogenes	Oranges (Hamlin)	6.0-6.5	4, 8 and 24	336, 336 and 24	3.9/4.0, 3.9/3.3 and 3.9/5.5	Pao et al., 1998
Listeria monocytogenes	Fruits salad	4.22	5	192	3.5/3.0	Mejía and Díaz, 1998
Listeria monocytogenes	Apple (Granny Smith)	4.1	20	96	4.5/≈ 4.5	Lanciotti et al., 2003
Listeria monocytogenes	Watermelon	5.50	10, 20 and 30	168, 48 and 24	2.5/6.0, 2.5/7.2 and 2.5/9.0	Penteado and Leitao, 2004
Listeria monocytogenes	Papaya	4.87	10, 20 and 30	168, 48 and 24	2.5/4.7, 2.5/4.3, and 2.5/7.3	Penteado and Leitao, 2004
Listeria monocytogenes	Melon	5.87	10, 20 and 30	168, 48 and 24	2.5/9.0, 2.5/9.0 and 2.5/9.0	Penteado and Leitao, 2004
Listeria monocytogenes	Pear (Cactus)	5.9	4, 8, 12 and 20	336	4.6/5.7, 4.6/7.6, 4.6/7.6 and 4.6/7.6	Corbo et al., 2005
Staphylococcus aureus	Oranges (Hamlin)	6.0-6.5	4, 8 and 24	336, 336 and 24	2.8/2.0, 2.8/2.0 and 2.8/3.5	Pao et al., 1998
Shigella flexneri	Watermelon	Not reported	22 - 26	6	2.8/4.5	Fernandez Escartin et al., 1989
Shigella spp.	Papaya	5.69	25 - 27	6	2.0 – 2.4/3.8 – 4.2	Fernandez Escartin et al., 1989

Table 4. Survival and growth of pathogenic bacteria in fruit juices

Pathogen	Juice type	pH	Temp. (ºC)	Storage time (hours)	Initial/final counts (Log CFU/g)	Reference
Escherichia coli O157:H7	Apple cider	3.6-4.0	25	72-144	5.0/<1.0	Zhao et al., 1993
Escherichia coli O157:H7	Apple cider	3.6-4.0	8	360-816	5.0/<1.0	Zhao et al., 1993
Escherichia coli O157:H7	Apple cider	3.6-4.0	8	264-360	2.0/<1.0	Zhao et al., 1993
Escherichia coli O157:H7	Apple cider	3.7-3.9	4	240	4.5/9.4	Miller and Kaspar, 1994
Escherichia coli O157:H7	Apple cider	3.5	21 and 4	168	5.3/<1.5 and 5.3/2.2	Uljas and Ingham, 1999
Escherichia coli O157:H7	Apple cider	3.7	26	144	6.0/4.5	Janisiewicz et al., 1999
Escherichia coli O157:H7	Apple cider	3.6-4.2	20-25	168	4.3/2.5-4.1	Dingman, 2000
Escherichia coli O157:H7	Apple juice	Not reported	8 and 25	336 and 72	5.1/5.0 and 5.3/5.1	Ceylan et al., 2004
Escherichia coli O157:H7	Apple cider	3.5-3.6	5	72	8.0/7.9	Inghan et al., 2006
Listeria monocytogenes	Apple juice	3.7	5 and 20	72 and 24	4.5/Nd and 4.5/Nd	Yuste and Fung, 2002
Listeria monocytogenes	Apple cider	3.5-3.6	5	72	5.8/1.5	Inghan et al., 2006
Salmonella spp.	Apple cider	3.5-3.6	5	72	7.9/6.4	Inghan et al., 2006
Salmonella ser. Enteritidis	Apple juice	4.2	35	24	5.0/3.01	Raybaudi-Massilia et al., 2006
Salmonella ser. Enteritidis	Pear juice	4.0	35	24	5.0/4.1	Raybaudi-Massilia et al., 2006
Salmonella ser. Enteritidis	Melon juice	5.9	35	24	5.0/7.9	Raybaudi-Massilia et al., 2006

The causal agents of microbiological spoilage in fruits and derivatives can be bacteria, as well as yeasts and molds. The latter are considered as the main spoilage group due to the low pH of most fruits. Nevertheless, some bacteria such as *Erwinia* spp., *Enterobacter* spp., *Pseudomonas* spp., and lactic acid bacteria have been reported as deteriorative in cut fruit and juices (Pao and Petracek, 1997; Brackett, 2001). Certain common molds such as *Penicillum* spp., *Aspergillus* spp., *Eurotium* spp., *Alternaria* spp., *Cladosporium* spp., *Paecilomyces* spp. and

Botrytis spp. have been shown to be involved in the spoilage of fresh fruit and some processed derivatives including the thermally processed (Splittstoesser, 1991; Beuchat and Pitt, 1992; Lund and Snowdon, 2000). On the other hand, Jay (2005) reported the occurrence of yeasts such as *Saccharomyces* spp. *Cryptococcus* spp. and *Rhodotorula* spp. in fresh fruits, and *Zygosaccharomyces* spp., *Hanseniaspora* spp., *Candida* spp., *Debaryomyces* spp. and *Pichia* spp. in dried fruits. Although both molds and yeasts are able to grow in the fruit tissue, the latter are more often associated with spoilage of cut fruits due to their ability to grow faster than molds (Jay, 2005).

Four types of factors determine the colonization of fresh-cut fruits and derivatives: 1) Intrinsic factors, which are dependent on the food composition, such as water activity, pH, redox potential, nutrients, structures and antimicrobial agents. 2) Technological treatments, which can modify the initial microflora. 3) Extrinsic factors or environmental conditions of the medium such as temperature, relative humidity and atmosphere. 4) Implicit factors, which depend on the developing microflora and the handling of the raw material and product (Montville and Matthews, 2001; Soliva-Fortuny and Martín-Belloso, 2003; Díaz-Cinco et al., 2005).

3- APLICATION OF NATURAL ANTIMICROBIALS IN FRESH-CUT FRUITS AND FRUIT JUICES

Food antimicrobials are chemical compounds added to, or present in foods that delay microbial growth or cause microbial death (Davidson and Zivanovic, 2003). The major targets for antimicrobials are food poisoning microorganisms (infective agents and toxin producers) and spoilage microorganisms, whose metabolic end products or enzymes cause off-odors, off-flavors, texture problems and discoloration (Davidson, 2001).

Most food antimicrobial agents are only biostatic but not biocidal. Therefore, their action on the food product is limited and shelf-life of the product will depend on the storage conditions. On the other hand, the use of combinations of antimicrobials could be preferred versus an antimicrobial alone since some microorganisms could be not inhibited or killed by the doses of antimicrobial substances that are legally approved or sensory accepted (Beuchat, 2001). The combined use of two or more antimicrobial compounds can result into synergistic, additive or antagonic effects. Similar results might be expected by combining their use with other preservation methods such as heat or pH modification. However, these combinations of

techniques must be tested for each specific food product before application in order to find desired synergies and avoid antagonistic effects (Wiley, 1994).

Antimicrobials generally have different concentration thresholds for inhibition or inactivation. These thresholds depend on the specific action targets of the antimicrobial substance, including cell wall, cell membrane, metabolic enzymes, protein synthesis, and genetic systems. The exact mechanism(s) or target(s) for food antimicrobials are often not known or well defined. It is difficult to identify a target when many interacting reactions take place simultaneously. For example, membrane-disrupting compounds could cause leakage of cellular content, interference with active transport or metabolic enzymes, or dissipation of cellular energy in ATP form (Davidson, 2001).

3.1- Factors affecting the antimicrobial activity

The preservative action of antimicrobials depends on the type, genus, specie, and strain of the microorganism tested. Efficiency of an antimicrobial also depends to a great extent on environmental factors such as pH, water activity (a_w), temperature, atmosphere, initial microbial load, and acidity of the food substrate (Gould, 1989; Wiley, 1994; Davidson, 2001). Many of these environmental factors can be considered individually as preservation methods when they are used at high doses, whereas the combined use of some of these treatments has been the basis of the hurdle concept which consists in the use of more than one treatment in a logical sequence to provide fresh-like quality food products (Wiley, 1994).

The antimicrobial nature of any compound is mostly determined by their chemical properties, outstandingly the pKa value, hydrophobicity/lipophilicity as measured by the partition coefficient log P_{oct}, solubility, and volatility, particularly in open systems (Stratford y Eklund, 2003). The pH is one of the more influential factors in the food antimicrobial agent effectiveness. Another factor affecting antimicrobial activity is the polarity of the substances. This relates to both the ionization of the molecule and the contribution of any alkyl side groups or hydrophobic parent molecules (Davidson, 2001). Therefore, it is very important to know the specific characteristics of the food system that needs to be preserved since a high proportion of lipids could limit the antimicrobial effectiveness of some antimicrobial agents as a consequence of the linkage between the food lipids and the antimicrobial substances with

hydrophobic characteristics. On the other hand, hydrophobic or partially hydrophobic characteristics of some antimicrobial substances difficult their dissolution in water and therefore they can not be used to prepare dipping solutions, which is a common technique in fresh-cut fruit processing.

3.2- Antimicrobials from animal origin

3.2.1-Enzymes

Lactoperoxidase is a hemoprotein present in milk and other secretions which catalyzes the oxidation of thiocyanate and iodide ions to generate highly reactive oxidizing agents. These products have a broad spectrum of antimicrobial effects against bacteria, fungi and viruses (Naidu, 2000). This enzyme is primarily active against H_2O_2-producing bacteria such as *Lactobacillus* and *Streptococcus* spp., although certain Gram negatives, catalase-positive organisms may also be inhibited. The lactoperoxidase system results in selective damage to the bacterial citoplasmic membrane without affecting mammalian cells (Beuchat and Golden, 1989).

Studies about the effect of enzymes on pathogenic or spoilage microorganisms naturally present or intentionally inoculated in fresh-cut fruits are not available in literature. However, their effects on pathogenic bacteria in fruit juices have been reported. In such sense, Van Opstal et al., (2006) inactivated *Escherichia coli* O157:H7 and *Shigella* spp. in freshly squeezed and pasteurized apple, orange, tomato and pink grape juices with peroxidase systems such as lactoperoxidase (LPER)-thiocyanate and soybean peroxidase (SBP)-thiocyanate. They concluded that LPER system, more than SBP system has interesting properties as biopreservative in acid juices. Reductions of ≥ 5 log CFU/ml of *E. coli* O157:H7 and *Shigella* spp. in freshly extracted and pasteurized apple juice stored at 6 and 20ºC for 24h were found using 30μg/ml of LPER. Addition of the same concentration in pasteurized orange juice resulted in reductions of 2 and 5 log CFU/ml in *E. coli* O157:H7 and *Shigella* spp. counts. Nevertheless, non significant activity against inoculated pathogens was reported in freshly extracted orange juice regardless the storage temperature, whereas a slight effect, leading to ≤1 log CFU/ml reductions of *Shigella* spp. was observed in pasteurized tomato juice stored during 24 h at 20ºC.

The use of other enzymes such as lysozyme to inactivate pathogenic microorganisms has also been reported for fruit juices.

Lysozyme is a protein present in milk and eggs that catalyzes the hydrolysis of the β-1,4 linkages between N-acetylmuramic acid and N-acetylglucosamine in the peptidoglycan layer of the bacterial cell wall. Lysozyme is active against most Gram positive bacteria, particularly thermophilic spore formers (Beuchat and Golden 1989). Hughey and Johnson (1987) reported that lysozyme is inhibitory to several food spoilage organisms as well as some pathogenic organisms, including *Listeria monocytogenes*, *Campylobacter jejuni*, *Salmonella* Typhimurium, *Bacillus cereus*, and *Clostridium botulinum*. Gram positive bacteria are more susceptible to lysozyme than Gram negative bacteria due to the different content in peptidoglycan in their cell walls, about a 90% in the former in front of only a 5-10% peptidoglycan in the latter. In addition, peptidoglycan layer in Gram negative bacteria lies beneath the outer membrane of the cell, difficulting the lysozyme action over this layer (Losso et al., 2000).

Liang et al., (2002) achieved 0.05, 0.08 and 1.3 log CFU/ml inactivation of *Salmonella* Typhimurium in freshly squeezed non-pulpy and pulpy orange juice and in pasteurized juice, respectively, using 0.1 µg/ml of lysozyme. However, those authors indicated that the combination of a Pulsed Electric Fields (PEF) treatment (30 pulses of 90 kV/cm) with lysozyme resulted in higher reductions in *Salmonella* Typhimurium counts (0.23, 0.20 and 2.48 log CFU/ml). In addition, it was demonstrated that a combination of lysozyme (0.1 µg/ml) with nisin (0.1 µg/ml) caused 0.1, 0.12 and 1.9 log CFU/ml reductions, whereas higher reductions of *Salmonella* Typhimurium (0.4, 0.36 and 4.2 log CFU/ml) were attained when lysozyme, nisin and PEF treatments were altogether used.

Wu et al., (2005) achieved a reduction of 5.2 log CFU/ml of the naturally occurring flora in intentionally spoiled pasteurized red grape juice by applying a combination of lysozyme (4 µg/ml), heat at 50ºC and a PEF treatment (20 pulses of 80 kV/cm). However, lower microbial reductions (4.2 log CFU/ml) were found by those authors when a combination 1:1 of lysozyme (4 µg/ml): nisin (4 µg/ml), heat at 50ºC and PEF (20 pulses of 80 kV/cm) were used. On the other hand, Liang et al., (2006) reported negligible changes (0.02 log CFU/ml inactivation) in the natural microbial population of intentionally spoiled freshly squeezed apple cider when combining 1:3 nisin (0.04 µg/ml):lysozyme (0.04 µg/ml), whereas 1.78 log CFU/ml inactivation was reached using the same combination of antimicrobials with a PEF treatment (17.6 pulses of 27kV/cm) at 50ºC.

3.2.2-Polysaccharides

Chitosan is a heteropolysaccharide composed of 1,4-linked 2 amino-2-deoxy-b-d-glucose obtained commercially by deacetylation of chitin, which is an abundant constituent of crustacean shells and fungi (Rhoades and Roller, 2000; Roller 2003; Sebti et al., 2005; Rodriguez et al., 2005). The general properties and applications of chitin, chitosan and their derivatives in foods have been studied extensively (Shahidi et al., 1999; No et al., 2007). However, its antimicrobial effect has been scarcely evaluated. In such sense, Rhoades and Roller, (2000) indicated that when chitosan is dissolved in saline, distilled water or laboratory media, it exhibits antimicrobial activity against some strains of filamentous fungi, yeasts and bacteria. In addition, Begin and Van Calsteren (1999) indicated that the positive charges of chitosan would interfere with the negatively charged residues of macromolecules at the cell surface, causing the membrane leaky.

The use of chitosan in edible coatings has been evaluated for cut fruit. Thus, González-Aguilar et al., (2005) reported that the incorporation of chitosan of low, medium and high molecular weight at concentrations of 1 and 2% (w/v) into edible coatings affected the growth of mesophilic bacteria and fungi in coated fresh-cut papayas stored at 5ºC for 15 days. A reduction of 3 Log UFC/g in mesophilic bacteria counts during the storage time was found when low (2% w/v) and medium (1 and 2% w/v) molecular weight chitosan coatings were used, whereas populations of yeast and moulds were inhibited throughout all the storage time. Likewise, Chien et al., (2007) found a substantial antimicrobial effect of an edible chitosan coating in concentrations of 0.5, 1.0 and 2% (w/v) in sliced mango stored at 6ºC, also observing that edible chitosan coatings retarded water loss and the drop in sensory quality, increasing the soluble solids content, titratable acidity, and ascorbic acid content.

The use of chitosan as antimicrobial agent against pathogenic microorganisms in fruit juices has not been reported. However, its use to control spoilage flora in fruit juices has been studied. In such sense, Roller and Covill, (1999) demonstrated that chitosan in a range of concentrations between 0.01 to 0.5 % (w/v) was effective to inhibit the growth of yeasts and moulds in apple juice, indicating that the inhibition and inactivation of filamentous moulds and yeasts was dependent on the chitosan concentration, pH and temperature. Likewise, Rhoades and Roller (2000) reported that the addition of 0.03 % (w/v) of chitosan to apple-elderflower

juice completely eliminated yeasts during 13 days of storage at 7ºC, while the total microbial counts and lactic acid bacteria counts increased at lower rates than those observed in non-treated samples.

3.3- Antimicrobials from plants origin

3.3.1-Essential oils, herbs and spices

Essential oils (EOs) also called volatile or ethereal oils are aromatic volatiles oily liquids obtained from plant material (flowers, buds, seeds, leaves, twigs bark, herbs, wood, fruits and roots) which can be obtained by fermentation, extraction or distillation, being this later method the most commonly used for commercial production of oils, that are constituted by a complex mix of compounds including terpenes, alcohols, cetones, phenols, acids, aldehydes and esters (Nychas, 1995; Burt, 2004; Ayala-Zavala et al., 2005). EOs are mainly used as food flavourings, in perfumes (fragrances and aftershaves), and as functional components in pharmaceuticals (Nychas and Skandamis, 2003). Individual components of EOs are also used as food flavorings, either extracted from plant material or synthetically manufactured (Burt, 2004). Although the majority of the EOs are classified as Generally Recognized As Safe (GRAS) substances (USFDA, 2006), their use in food as preservatives is often limited due to flavor considerations (Lambert et al., 2001). Many herbs and plant extracts possess antimicrobial activities against a wide range of bacteria, yeasts and moulds (Conner, 1993; Kim et al., 1995; Beuchat, 2001; Friedman et al., 2002; Burt, 2004; Holley and Patel, 2005; Rojas-Graü et al., 2006). However, most of them appear to be more effective in laboratory media than in real foods (López-Malo et al., 2000; Nychas and Skandamis, 2003).

Although the antimicrobial properties of EOs and their components have been reviewed in the past, the mechanism of action has not been studied in great detail. Considering the large number of different groups of chemical compounds present in EOs, it is most likely that their antibacterial activity is not attributable to one specific mechanism but to the action over several specific targets in the cell. López-Malo et al., (2000), Nychas and Skandamis (2003) and Burt (2004) have reported the location and mechanisms of action in the bacterial cell of EOs, for instance: degradation of the cell wall, damage to cytoplasmic membrane, damage to membrane proteins, leakage of cell contents, coagulation of cytoplasm and depletion of the proton motive force (Fig. 1). These authors have also

suggested that these mechanisms are not working as independent targets; some of them are affected as a consequence of another mechanism being targeted. Likewise, Nychas and Skandamis (2003) indicated that the mode of action of EOs is concentration dependent, indicating that low concentrations inhibit enzymes associated with energy production while higher amounts may precipitate proteins. In addition, Ayala-Zavala et al., (2005) pointed out that the antimicrobial effects of EOs depend on their hydrophobicity and migration through the microbial cytoplasmic membrane.

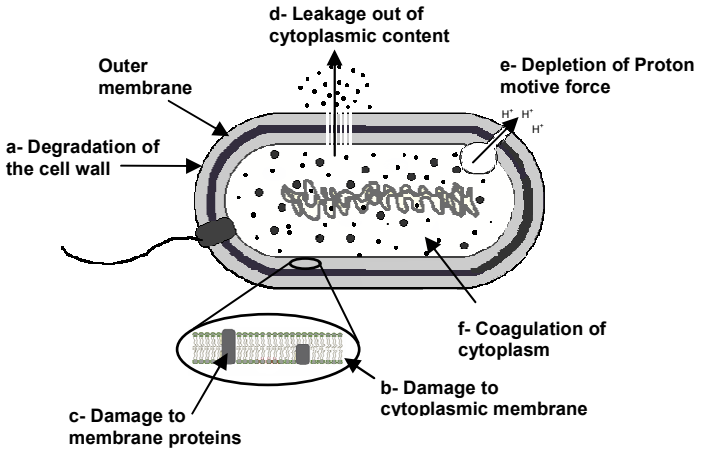

Figura 1.- Mechanisms of action of the essential oils and their components in bacterial cell (a-f). The amplification illustrates the action mode over the inner membrane.

Lambert et al., (2001), Gill and Holley (2006) and Oussalah et al., (2006) reported that oregano oil, savory oil, thymol, eugenol and carvacrol caused disruption of the cellular membrane, inhibition of the ATPase activity and release of the intracellular ATP of *E. coli*, *E. coli* O157:H7, *L. monocytogenes, Lactobacillus sakei, Pseudomonas aeruginosa* and *Sthaphylococcus aureus*. Likewise, Raybaudi-Massilia et al., (2006) observed disruption of the cellular membrane with leakage out of the cytoplasm content in *S.* Enteritidis when lemongrass oil was used. On the

other hand, Oussalah et al (2006) and Gill and Holley (2004, 2006) reported a decrease in the intracellular ATP by ATPase activity without apparent changes on the cell membrane of *E. coli*, *E. coli* O157:H7 and *L. monocytogenes* when cinnamon oil and cynnamaldehyde were used. In such sense, a mechanism for cynnamaldehyde action was suggested, in which interaction with the cell membrane causes disruption sufficient to disperse the proton motive force by leakage of small ions without leakage of large cell components such as ATP. In addition, Wendakoon and Sakaguchi (1995) mentioned a possible action of cynnamaldehyde on the embedded proteins in the cytoplasmic membrane of *Enterobacter aerogenes* by inhibition of amino acid decarboxylase enzymes, which are necessary for the amino acid biosynthesis and biodegradation.

Several authors have studied the effect of EOs over inoculated microorganisms used as pathogen indicators in fresh-cut fruit. Lanciotti et al., (2004) reported that the addition of 0.02% (v/v) of citrus, mandarin, cider, lemon and lime EOs to fresh sliced fruit mix (apple, pear, grape, peach and kiwifruit) increased the death rate of the inoculated *E. coli* population. On the other hand, Rojas-Graü et al., (2007) indicated that the incorporation of EOs such as lemongrass (1.0-1.5% v/v) and oregano (0.1-0.5% v/v) into an apple puree-alginate edible coating significantly reduced the inoculated population of *L. innocua* (used as model of *L. monocytogesnes*) in fresh-cut apple.

The antimicrobial effect of EOs over the naturally occurring flora of fresh-cut fruit has also been reported. In this way, Roller and Seedhar (2002) demonstrated that carvacrol and cinnamic acid (0.015% v/v) were effective in reducing and inhibiting the natural flora growth in fresh-cut kiwifruit and honeydew melon, respectively, without detrimental sensory effects. In contrast, higher concentrations 0.075-0.225% (v/v) of carvacrol were more effective to reduce the natural microflora of fresh-cut kiwifruit, although undesirable colour and odour changes were observed. On the other hand, Lanciotti et al., (2004) reported that citrus, mandarin, cider, lemon and lime EOs increased the shelf-life of minimally processed fruit salads without altering the sensory properties, even when the product was inoculated with spoilage microorganisms. The addition of 0.02% (v/v) of those substances to a fresh sliced fruit mix (apple, pear, grape, peach and kiwifruit) inhibited the proliferation of natural occurring microbial flora and reduced the growth rate of inoculated *Saccharomyces cerevisiae* population. In the same way, Ayala-Zavala et al., (2007) demonstrated that garlic oil in a concentration of 0.0333 % (v/v) applied as vapor effectively suppressed the microbial proliferation on fresh-cut tomato stored at 5ºC for

15 days. On the other hand, Rojas-Graü et al., (2007) found that the addition of EOs of lemongrass (1.0-1.5% v/v) and oregano (0.1-0.5% v/v) to an apple puree-alginate edible coating was effective to inhibit the naturally occurring flora of fresh-cut apples. However, changes in sensory characteristics such as colour and firmness were reported at the assayed concentrations.

Different studies have showed the effectiveness of EOs on foodborne pathogens in fruit juices. Raybaudi-Massilia et al., (2006) reported more than 5 log CFU/ml reductions of *L. innocua, S.* Enteritidis and *E. coli* in sterilized apple and pear juices containing 0.2% (v/v) of cinnamon leaf oil, lemongrass oil or geraniol incubated at 35ºC for 24h. However, higher concentrations of cinnamon oil (0.8% v/v), lemongrass oil (0.5% v/v) and geraniol (0.6% v/v) were required to inactivate those microorganisms in sterilized melon juice. In the same way, Friedman et al., (2004) reported that cinnamon leaf and cinnamon bark oils were considered among the 10 most active compounds against *S. enterica* serovar Hada in fresh apple juice together with melissa oil, carvacrol, oregano oil, terpeineol, geraniol, lemon oil, citral, lemongrass oil and linalool, with effective concentrations ranging between 0.0044-0.011% (v/v). Likewise, cinnamon leaf and cinnamon bark oils were included among the 10 most active compounds for reducing *E. coli* O157:H7 in fresh apple juice together with carvacrol, oregano oil, geraniol, eugenol, citral, clove bud oil, lemongrass oil and lemon oil. The effective concentrations of those ten compounds to reduce the bacterial population of *E. coli* O157:H7 in 60 min until 50% ranged between 0.018-0.093% (v/v). In addition, Friedman et al., (2004) pointed out that the bactericidal activity of different EOs or their active components against *E. coli* O157:H7 and *S. enterica* in apple juice incubated at 37ºC was in most cases about three times greater than at lower temperatures (4 and 21ºC). A greater fluidity of the microbial cell membrane at high temperatures could explain an increased cellular diffusion of antimicrobial substances. In such sesnse, Aronsson and Rönner (2001) indicated that the temperature of the medium in which cells are suspended has a significant influence in determining the membrane fluidity properties. At low temperatures, the phospholipids are closely packed into a rigid gel structure, while at high temperatures they are less ordered and membrane has a liquid-crystalline structure.

On the other hand, the effect of EOs against naturally occurring microflora has also been studied. Liang et al., (2006) reported only 0.06 and 0.07 log CFU/ml reductions in the inoculated native flora of freshly squeezed apple cider using 3 and 5 % (v/v) clove oil suspensions,

respectively. However, 2.88 and 3.11 log CFU/ml reductions were reached by combining the use of clove oil with a PEF treatment (17.6 pulses of 27kV/cm) at 50ºC. Likewise, Nguyen and Mittal, (2007) found a reduction of 3.9 log CFU/ml in the inoculated native flora of pasteurized tomato juice when 0.1% (v/v) clove oil was used in combination with heat at 50ºC.

Mint family contains many different types including peppermint (*Mentha piperita* L.) and spearmint (*Mentha spicata*). Some of their active constituents are menthol, menthone, carvone and others monoterpenes. The antimicrobial properties of mint against pathogenic microorganisms have been reported (Tassou et al., 1995 and 2000). However, the use of mint extract to control pathogenic and deteriorative microorganisms in fresh-cut fruits has not been reported in literature. Indeed, scarce information is available on fruit juice applications. Nguyen and Mittal (2007) reported 4.77 and 8.34 log CFU/ml reductions in the native flora of pasteurized tomato juice intentionally spoiled when mint crystals at 0.1 and 1.2 % (w/v) were used in combination with heat at 50ºC.

Vanillin (4-hydroxy-3methoxybenzaldehyde) is the predominant phytochemical present in vanilla beans and is a GRAS flavouring compound widely used in ice cream, beverages, biscuits, chocolate, confectionary, desserts, etc. (Vasantha-Rupasinghe et al., 2006). Based on the studies conducted with *E. coli*, *Lactobacillus plantarum* and *L. innocua* the inhibitory activity of vanillin resides primarily in its ability to negatively affect the integrity of the cytoplasmic membrane, with loss of ion gradients, pH homeostasis and inhibition of respiratory activity, maintaining the energy generation largely unaffected (Fitzgerald et al., 2004a).

The bactericidal activity of vanillin on *L. innocua* inoculated on fresh-cut apples coated with an apple puree-alginate edible layer was studied as a model for *L. monocytogenes* (Rojas-Gräu et al., 2007). Significant reductions (3 log CFU/g) of *L. innocua* populations were found in coated apple pieces compared to control samples when vanillin (0.3 and 0.6% w/v) was incorporated into the edible coating formulations. The incorporation of vanillin was also effective in inhibiting the growth of psychrophilic bacteria and fungi on apple pieces, with maximal populations below to 10^4 CFU/g after 21 days of refrigerated storage. In the same way, Vasantha-Rupasinghe et al., (2006) demonstrated that the incorporation of vanillin (0.18% w/v) into dipping treatments for apple slices inhibited microbial growth during 19 days of storage by 37 and 66% in “Empire” and “Crispin” apple slices, respectively.

Vanillin has also a potential use to prevent the spoilage of fruits juices and purees. In such sense, Cerruti et al., (1997) evaluated the use of vainillin as natural antimicrobial for producing shelf-stable strawberry puree. Growth of both native (aerobic, anaerobic mesophilic and yeast and moulds) and inoculated flora (*Saccharomyces cerevisiae*, *Zygosaccharomyces rouxii*, *Z. bailii*, *Schizosaccharomyces pombe*, *Pichia membranaefaciens*, *Botrytis* sp., *Byssochlamys fulva*, *Bacillus coagulans* and *Lactobacillus delbrueckii*) was prevented for more than 60 days of storage at room temperature in pasteurized strawberry puree containing 0.3% (w/v) vanillin. On the other hand, Fitzgerald et al., (2004b) reported that concentrations of 0.30 and 0.15% (v/v) vanillin in a pasteurized apple juice and a peach-flavored soft drink, respectively, were required to achieve the complete inhibition of both inoculated *S. cerevisiae* and *Candida parapsilosis* at 25ºC over an 8 week storage period. Likewise, those authors indicated that the effective levels of vainillin were 0.075 and 0.015% (v/v) when the storage temperature was reduced to 8ºC.

Cinnamon powder obtained from bark is widely used as a spice with antioxidant and antimicrobial activities. It contains cinnamaldehyde and eugenol as major compounds responsible for the antimicrobial effect. The use of this spice to control pathogenic and spoilage flora in fresh-cut fruits has not been reported. However, its use to inactivate pathogenic microorganisms such as *L. monocytogenes* and *E. coli* O157:H7 in fruit juices has been studied. Yuste and Fung (2002) reported that 4-6 log CFU/ml reductions of *L. monocytogenes* were attained in pasteurized apple juice with 0.1, 0.2 and 0.3% (w/v) of ground cinnamon after 1h of incubation at 5 and 20ºC. In addition, no growth of the microorganism occurred during 7 days of storage. On the other hand, Ceylan et al., (2004) demonstrated that the addition of 0.3% (w/v) cinnamon into pasteurized apple juice gradually decreased the number of *E. coli* O157:H7 in 1.6 (8ºC) and 2.0 (25ºC) log CFU/ml after 14 and 3 days, respectively. In contrast, Iu et al. (2001) reported an immediate 2 log CFU/ml reduction of *E. coli* O157:H7 in unpasteurized apple cider maintained at 42ºC by adding 2% cinnamon powder.

3.3.2-Aldehydes

Aldehydes are dominant compounds released by plant tissue through the lipoxygenase pathway after some damage (Lanciotti et al., 1999). The precise action mode is not yet clear but passive diffusion across the plasma membrane is likely to occur. Once inside cells, aldehydes would

react with nucleophilic groups playing a key role in living cells, namely sulfydryl groups present in proteins and lower molecular weight compounds such as glutathione. Although the precise targets in microbial cells remain unclear, the toxicity of these molecules seems to be dependent on the membrane phospholipidic bilayer affinity (Lanciotti et al., 2003). The antimicrobial activity against pathogenic and spoilage species of some aldehydes such as hexanal and (E)-2-hexenal, which are components of the aroma of many fruits and vegetables has been demonstrated. Lanciotti et al., (2003) reported significant extensions of the lag phase of *E. coli* and *Salmonella* Enteritidis in apple slices treated with hexanal (150 μl/l), hexyl acetate (150 μl/l) and (E)-2-hexenal (20 μl/l) stored at 20ºC, whereas for *L. monocytogenes* a bactericidal effect after 4 days was found under the same experimental conditions.

On the other hand, Lanciotti et al., (1999) reported that the use of 0.225 μl/l hexanal prolonged the lag phase of native yeasts for 8 days on sliced apple stored at 15ºC under modified atmosphere, whereas mesophilic bacteria growth was retarded by more than 20 days in the same conditions. Likewise, Corbo et al., (2000) attained an extension of 12 days in the lag phase of an inoculated spoilage yeast (*Pichia subpelliculosa*) on sliced apple stored at 15ºC under modified atmosphere when using 0.3μl/l of hexanal, whereas 0.06 μl/l of trans-2-hexenal extended the lag phase up to 5 days. In addition, antimicrobial activity of hexanal and trans-2-hexenal was influenced by the vapour pressure of the active compounds, which is temperature-dependent.

3.3.3-Organic acids

Organic acids have been traditionally used in the food industry as preservative agents since pH is one of the parameters governing the survival and growth of microorganisms in food. The alteration of the hydrogen ion concentration influences the growth or inhibition of a microorganism. In general, bacteria prefer a pH near to neutrality (pH 6.5-7.5), but tolerate a pH range of 4-9. Yeasts are more tolerant than bacteria to low pH values, whereas molds can growh over the widest range of pH. Therefore, one effective mean of limiting microbial growth is to increase the acidity of a food by either adding an acidifier or enhancing natural fermentation to develop a change in acidity (Doores, 1993).

Given the metabolic complexity of the microbial cell, either prokaryote (bacteria) or eukaryote (yeasts and moulds), it is very unlikely for a chemical compound to affect a single site of action only. Organic

acids can thus likely affect a number of systems in the target organism. The effect on each point of action will depend in turn on variables such as acid type and concentration, conditions of use, pH, temperature, and nature of the target organism. This may explain in some way the contradictory theories and publications concerning the antimicrobial actions of organic acids (Stratford and Eklund, 2003). Possible mechanisms of action of organic acids (Fig. 2) include: direct pH reduction of the substrate or growth medium due to an increase of proton concentration, depression of the internal cellular pH by ionization of the undissociated acid molecule, or disruption of substrate transport by alteration of cell membrane permeability. In addition to inhibiting substrate transport, organic acids may also inhibit NADH oxidation, thus eliminating supplies of reducing agents to electron transport systems (Beuchat and Golden, 1989; Beuchat, 2000; Davidson, 2001). Because the undissociated portion of the acid molecule

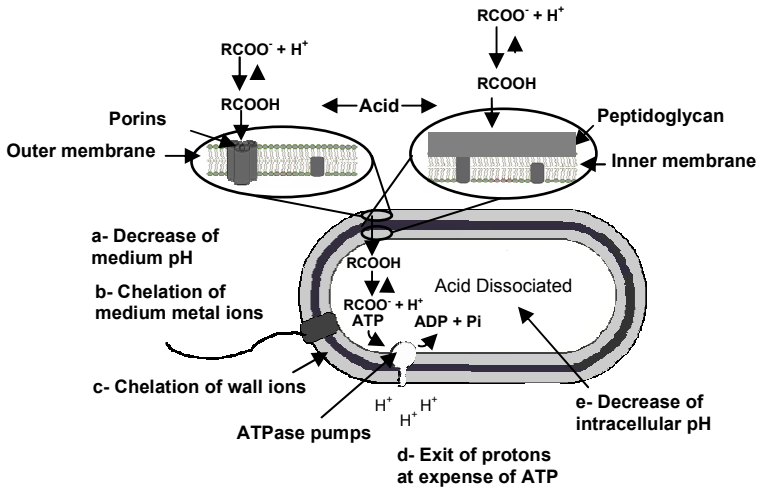

Figura 2.- Mechanisms of action of the organic acids in bacterial cell (a-e). The left amplification illustrate how the organic acids can pass through the outer membrane in Gram-negative bacteria, whereas the right amplification show how they can pass through the inner membrane in Gram-positive.

is primarily responsible for antimicrobial activity, effectiveness at a given pH depends largely on the dissociation constant (pKa) of the acid (Beuchat, 2000; Davidson, 2001). Fully dissociated "strong" acids such as hydrochloric or sulphuric acids affect microbes only through alteration of pH (proton concentration), since chloride or sulfate concentrations appear to have little effect. However, when media are acidified with "weak" acids, such as citric, acetic, or lactic acids, the antimicrobial effects are more pronounced, indicating that "weak" acids inhibit microbes by other mechanisms additional to that of merely lowering pH (Stratford and Eklund, 2003). Undissociated form of organic "weak" acids can penetrate the cell membrane lipid bilayer more easily. Once inside the cell, the acid is forced to dissociate into charged anions and protons because the cell interior has a higher pH than the exterior. Protons generated from intracellular dissociation cause progressive decline in intracellular pH which in turn may inhibit glycolisis, affect cell signaling, and inhibit active transport (Stratford and Eklund, 2003). Bacteria have to exclude the protons generated to the cellular exterior to prevent conformational changes in the cell structural proteins, enzymes, nucleic acids, and phospholipids. According to the chemiosmotic theory, the cytoplasmic membrane is impermeable to protons, and they must be transported to the exterior implying an energetic cost in the ATP form which will eventually deplete the cellular energy (Davidson, 2001). Organic acids also interfere with membrane permeability. Thus, Sheu and Freese, (1972) suggested that short-chain organic acids interfere with energy metabolism by alteration of the structure of the cytoplasmic membrane due to an interaction with membrane proteins.

Different studies about the effect of organic acids direct or indirectly added to fresh-cut fruits and fruit juices over pathogenic and spoilage microorganisms have been carried out. In this way, DiPersio et al., (2003) achieved 0.7 and 0.9 log CFU/g reductions in the population of *Salmonella* inoculated in "Gala" apple slices treated for 10 min with solutions containing 3.40% (w/v) ascorbic acid and 0.21% (w/v) citric acid. Similar results were found by Derrickson-Tharrington et al., (2005) for *E. coli* O157:H7 inoculated on "Gala" apple slices after a dipping treatment (10 min) in acidic solutions of ascorbic acid (2.8% w/v), citric acid (1.7% w/v) and commercial lemon juice (50% v/v), reporting reductions of 1.3, 1.3 and 1.2 log CFU/g in *E. coli* O157:H7 counts, respectively. However, higher reductions of *Salmonella* and *E. coli* O157:H7 were reported in those studies when samples were dried for 6h at 60º and 62.8ºC, respectively. Up to 5.2 and 3.8 log CFU/g reductions of *Salmonella* were

reached when ascorbic acid and citric acid were used, whereas counts of *E. coli* O157:H7 were reduced up to 6.7, 6.7 and 7.1 log CFU/g when ascorbic acid, citric acid and lemon juice were applied, respectively. Likewise, Castillo and Escartín, (1994) found significant reductions of 85.7 and 100% inoculated *Campylobacter jejuni* in watermelon and papaya cubes, respectively, treated with lemon juice (0.05 ml per cube of 4 cm^2 side) and stored for 6h at room temperature (25-29ºC).

Organic acids have also been incorporated into dipping solutions to reduce or inhibit the native flora in fresh-cut fruits. Pao and Petracek, (1997) extended the shelf-life of peeled or chunked oranges using a solution of citric acid at different concentrations. The maximal inhibition of spoilage bacteria was attained with 0.5% (w/v) of citric acid for storage at 4ºC and 1% (wtv) for storage at 8 and 21ºC regardless the treatment method (dipping or infusion). On the other hand, Raybaudi-Massilia et al., (2007) inhibited the growth of mesophilic and psychrophilic bacteria and fungi in fresh-cut apples by adding malic acid (2.5% w/v) into a dipping solution containing calcium lactate (1% w/v), N-acetyl-L-cysteine (1% w/v) and glutathione (1% w/v) as stabilizing substances. A reduction in the growth rate and an increase in the lag phase of microbial populations were observed during storage of apple pieces at 5ºC, thus leading to an extension of the microbiological shelf-life by at least 23 days. However, the shelf-life was limited due physicochemical changes to 14 days in those cut apples.

The use of organic acids added to fruit juices, alone or in combination with other preservation methods has been studied as an alternative for thermal pasteurization processes. Important reductions of pathogenic microorganisms such as *E. coli* O157:H7, *Salmonella* serovars and *L. monocytogenes* have been attained in fruit juices by adding acid fruit juices and organic acids. Uljas and Ingham (1999) reported reductions of ≥ 5 log CFU/ml of *E. coli* O157:H7 and *S.* Typhimurium DT104 in unpasteurized apple cider (pH 3.3 and 3.7) stored for 6 h at 35ºC. However, 0.1% (w/v) sorbic acid was required when cider pH was 4.1. In contrast, lower reductions of those pathogenic microorganisms were reached when 1% (v/v) lactic acid or 1% (w/v) propionic acid were used. Reductions of ≥ 5 log CFU/ml of *E. coli* O157:H7 and *S.* Typhimurium were also achieved in unpasteurized apple cider (pH 4.1) stored at 35ºC for 6 and 4h, respectively, after a freezing-thawing treatment (-20ºC 48h; 4ºC 4h); however, when a combination of sorbic acid with freezing-thawing treatment were applied, a 5 log CFU/ml decrease in *E. coli* O157:H7 was attained in lower time (4h).

On the other hand, Comes and Beelman, (2002) evaluated the effectiveness of several acidulants such as fumaric, citric and malic acids, added at 0.10% (w/v) against *E. coli* O157:H7 populations in unpasteurized apple cider treated at 45ºC for 20 min and stored for 48h at 4ºC. Fumaric acid was found to reduce *E. coli* O157:H7 populations by 3.6 log CFU/ml, whereas citric and malic acids did not result in significant reductions (<1.3 log CFU/ml reductions) compared to the control cider. Furthermore, the effectiveness of fumaric acid increased with increasing concentrations (0.10, 0.15 and 0.20% w/v) at lowest pH as a consequence of the acid pKa. In the same way, Chikthimmah et al., (2003) reported >5 log CFU/ml reductions in *E. coli* O157:H7 populations in unpasteurized apple cider stored at 5ºC during <60h using a combination of fumaric acid (0.15% w/v) and sodium benzoate (0.05% w/v). An increase in the storage temperature, from 15 to 25ºC, significantly reduced the time necessary to attain a 5 log CFU/ml inactivation from <20 to >5h. In addition, they indicated that an increase of apple cider pH caused a decrease in the destruction rate of *E. coli* O157:H7.

Ingham et al., (2006) indicated that the simple addition of 10 or 15% (v/v) cranberry juice to apple cider did not result in appreciable die-off of *E. coli* O157:H7 and *Salmonella* serovars, whereas *L. monocytogenes* population was reduced by 2.2 and 2.9 log CFU/ml additional cycles to those observed in apple cider alone (2.2 log CFU/ml), thus achieving more than 5 log CFU/g reductions when 15% of cranberry juice was added to the apple cider. In addition, reductions of 1.5 and 3 log CFU/ml of *Salmonella* serovars were reached in apple cider and apple cider with 10 or 15% (v/v) of cranberry juice, respectively, after 3 days at 5ºC; whereas slight reductions of *E. coli* O157:H7 (0.1-0.4 log CFU/ml) were reported under those same conditions. On the other hand, those authors demonstrated that > 5 log CFU/ml reductions in *L. monocytogenes* and *Salmonella* were achieved by combining the addition of cranberry juice at 10% (v/v) and mild temperatures (45ºC for 2h) or mild temperatures and freeze-thaw (-20ºC for 24h, 5ºC for 24h). Nevertheless, those combinations were not enough to attain 5 log CFU/ml reductions of *E. coli* O157:H7.

Control of native microflora in fruit juices using organic acids have also been reported by Comes and Beelman, (2002) who reduced the growth rate of the naturally occurring aerobes by using a combination of fumaric acid (0.15% w/v) with sodium benzoate (0.05% w/v) in unpasteurized apple cider held at 25ºC for 6h followed by refrigeration at 4ºC for 28 days.

3.3.4- Esters

Methyl jasmonate is a natural compound derivate of jazmine (Ayala-Zavala, et al., 2005), which is found as a lipid of the plant cell membranes, synthesized via the lipoxygenase pathway (Ippolito and Nigro, 2003). Some of its properties are: to regulate the plant growth, to promote the stomas closing, to act as second messenger and decrease the pathogens attack (Ayala-Zavala, et al., 2005).

Literature reporting the use of methyl jasmonate in fresh-cut fruits to control pathogenic microrganisms was not found. On the other hand, although different studies have been published about the effectiveness of methyl jasmonate to reduce the spoilage of whole products from vegetable origin (Ayala-Zavala, et al., 2005), few researchers have proposed the use of this compound to control the spoilage flora of fresh-cut fruit. In this way, Wang and Buta, (2003) reported that methyl jasmonate in concentrations of 11.2 and 22.4 µl/l applied as vapor resulted effective for preventing mold growth in fresh-cut kiwifruit during 3 weeks of storage at 10ºC. Likewise, Martínez-Ferrer and Harper, (2005) reached reductions of 3 log CFU/g of the native microflora on fresh-cut pineapple after 12 days of storage at 7ºC using an emulsion of methyl jasmonate in a concentration of 15 µl/l. In addition, those authors indicated that methyl jasmonate in the same concentration but applied as vapor resulted less effective in reducing the microbial population. Consistently, Ayala-Zavala et al., (2007) demonstrated that methyl jasmonate in a concentration of 22.4 µl/l applied as vapor suppressed microbial proliferation in fresh-cut tomato stored at 5ºC for 15 days. However, a combination of methyl jasmonate (22.4 µl/l) and ethanol (300 µl/l) applied as vapor resulted more effective in inhibiting the microbial growth along the storage time than the individual treatments with each volatile compound.

3.4- Antimicrobials from microbial origin

Nisin is a small, heat stable antimicrobial peptide of 34 amino acid produced by *Lactococcus lactis* subsp. *lactis* (Davidson and Zivanovic, 2003) which has been described as a class 1 bacteriocin, a group that comprises lantibiotics which are a family of membrane active peptides containing the unusual thioether amino acids lanthionine and β-methyl lanthionine as well as other modified amino acids, such as dehydrated serine and threonine. Nisin has shown a narrow antimicrobial spectrum, inhibiting only Gram-positive bacteria, including *Alicyclobacillus*, *Bacillus*

cereus, *Brochothrix thermosphacta*, *Clostridium botulinum*, *C. sporogenes, Desulfotomaculum*, *Enterococcus*, *Lactobacillus*, *Leuconostoc*, *Listeria monocytogenes*, *Micrococcus*, *Pediococcus*, *Sporolactobacillus*, and *Staphylococcus*. Against bacterial spores, nisin is sporostatic rather than sporicidal. On the other hand, nisin does not generally inhibit Gram-negative bacteria, yeast or moulds. The activity spectrum includes Gram-negative bacteria when used in combination with chelating agents (e.g.,EDTA). Nisin activity generally increases at low pH and low initial microbial loads (Davidson and Zivanovic, 2003; Ross et al., 2003).

In vegetative cells the primary site of action for nisin is the cytoplasmic membrane where nisin forms pores, destroys the membrane integrity and acts as a voltage dependent polarizer (Abee et al., 1994; Crandall and Montville, 1998; Ross et al., 2003). Pore formation results in depletion of proton motive force and loss of cellular ions, amino acids, and ATP (Crandall and Montville, 1998; Davidson and Zivanovic, 2003). Other action mechanisms against vegetative cells have been proposed for nisin, including interference with cell wall biosynthesis, although some authors have indicated that this may simply be a consequence of energy loss and membrane depolarization resulting from pore formation and induction of autolysis (Bierbaum and Sahl, 1987; Thomas et al., 2000).

The effectiveness of nisin used alone against pathogenic microorganisms in fresh-cut fruits has not been found in literature. However, Ukuku and Fett (2004) reported reductions of 1 and 1.4 log CFU/g *Salmonella* in fresh-cut cantaloupe melon using combinations of nisin (50 µg/ml)-sodium lactate (2% v/v)-potassium sorbate (0.02% v/v) and sodium lactate (2% v/v)-potassium sorbate (0.02% v/v), respectively.

On the other hand, Ukuku and Fett, (2002) reached 2 log CFU/g reductions in the mesophilic aerobic and lactic acid bacteria populations on fresh-cut cantaloupe melon after washing with a solution containing 10 µg/ml nisin and 0.02M EDTA. Nevertheless, those authors indicated that the growth of Gram-negative bacteria such as Pseudomonas and yeasts and moulds during 15 days of storage at 5ºC was not inhibited with that dipping treatment.

The effect of nisin alone or in combination with other preserving methodologies over pathogenic microorganisms in fruit juices has been evaluated by several researchers. Liang et al., (2002) inactivated 0.1, 0.1 and 1.5 log CFU/ml of *Salmonella* Typhimurium in non-pulpy, pulpy and pasteurized freshly squeezed orange juice, respectively, by adding 0.1 µg/ml nisin. However, those authors found that the use of a combination of a PEF treatment (30 pulses of 90kV/cm) with nisin resulted in higher

reductions in *Salmonella* Typhimurium counts (0.25, 0.20 and 2.95 log CFU/ml). Likewise, Iu et al. (2001) achieved a 4.63 log CFU/ml reduction of *E. coli* O157:H7 in unpasteurized apple cider using 20 µg/ml nisin; whereas a higher reduction (8.78 log CFU/ml) was achieved when a PEF treatment (10 pulses of 80kV/cm) and nisin were combined.

On the other hand, the use of nisin in combination with other preservation methods has been shown to be effective to control naturally occurring microbes in fruit juices. Thus, Wu et al., (2005) reported a 6.2 log CFU/ml reduction of naturally occurring flora of pasteurized red grape juice intentionally spoiled when applying a combination of nisin (4 µg/ml), heat at 51ºC and PEF (20 pulses of 80 kV/cm). On the other hand, Nguyen and Mittal, (2007) achieved a 0.85 log CFU/ml reduction in the naturally occurring microflora of intentionally spoiled pasteurized tomato juice by applying a combination of nisin (4 µg/ml) with a thermal treatment at 50ºC. Nonetheless, those authors found a higher reduction (4.4 log CFU/ml) of the natural microflora when a combination of nisin, heat at 50ºC and PEF (20 pulses of 80kV/cm) was used.

4- FINAL REMARK

The information compiled in this work demonstrates that different natural antimicrobials from animal, plant and microbial origin, direct or indirectly added to fresh-cut fruits and fruit juices can effectively reduce or inhibit pathogenic and spoilage microorganisms, thus representing a good alternative to their preservation. However, the addition of antimicrobials to these products without adversely affecting the sensory characteristics is still a challenge for researchers, since the concentrations of antimicrobials necessary to ensure safety (up to 5 log CFU/g reductions of pathogenic microorganisms based on USFDA regulation, 2002) of fresh-cut fruits and fruit juices are several times higher than those accepted by the consumers from a sensory point of view. Therefore, new studies about the combination of antimicrobials with other methodologies of food preservation are necessary to reduce the impact of the antimicrobials on the sensory properties.

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OBJETIVOS

OBJETIVO GENERAL

El objetivo general de esta tesis fue evaluar la efectividad de diferentes sustancias naturales antimicrobianas para controlar microorganismos patógenos y extender la vida útil de frutas frescas cortadas. Para tal fin fue necesario realizar estudios para determinar las concentraciones mínimas inhibitorias y mínimas bactericidas de las sustancias antimicrobianas seleccionadas contra microorganismos patógenos como *Listeria monocytogenes*, *Salmonella* Enteritidis y *Escherichia coli* O157:H7 en zumos de frutas para luego evaluar su efectividad contra esos microorganismos patógenos en frutas frescas cortadas, así como también para la extensión de la vida útil del producto. La efectividad de esas sustancias en frutas frescas cortadas fue evaluada en forma directa a través de tratamientos de inmersión o indirecta a través de la incorporación de estas sustancias en recubrimientos comestibles.

OBJETIVOS ESPECÍFICOS

1- Evaluar la actividad antimicrobiana de sustancias naturales en zumos de manzana, pera y melón.

a- Determinar las concentraciones mínimas inhibitorias (CMI) y mínimas bactericidas (CMB) de ácido málico contra *L. monocytogenes, S. Enteritidis* y *E. coli* O157:H7.

b- Determinar las concentraciones mínimas inhibitorias (CMI) y mínimas bactericidas (CMB) de aceites esenciales contra *L. innocua*, *S. Enteritidis* y *E. coli.*

2- Evaluar la actividad antimicrobiana de sustancias naturales aplicadas directamente a manzanas y peras frescas cortadas.

a- Determinar el efecto de ácido málico en combinación con estabilizadores de textura y color sobre *L. monocytogenes, S.* Enteritidis y *E. coli* O157:H7 en manzanas y peras frescas cortadas.

b- Determinar la vida útil de manzanas y peras frescas cortadas tratadas con ácido málico y estabilizadores de textura y color.

3- Evaluar la actividad antimicrobiana de sustancias naturales incorporadas en recubrimientos comestibles en manzana y melón frescos cortados.

a- Determinar el efecto de la incorporación de ácido málico y aceites esenciales en un recubrimiento comestible a base de alginato sobre *E. coli* O157:H7 y *S.* Enteritidis en manzana y melón frescos cortados.

b- Determinar la vida útil de manzana y melón frescos cortados cubiertos con un recubrimiento comestible a base de alginato con ácido málico, aceites esenciales y estabilizadores de textura y color incorporados.

Antimicrobial activity of malic acid against Listeria monocytogenes, Salmonella Enteritidis and Escherichia coli O157:H7 in apple, pear and melon juices

Raybaudi-Massilia, R. M., Mosqueda-Melgar, J. and Martín-Belloso, O.

Food Control (Enviado)

ABSTRACT

Minimal inhibitory (MIC) and minimal bactericidal (MBC) concentrations of malic acid against *L. monocytogenes*, *S.* Enteritidis and *E. coli* O157:H7 inoculated in apple, pear and melon juices stored at 5, 20 and 35ºC were evaluated. MICs and MBCs against *L. monocytogenes, S.* Enteritidis and *E coli* O157:H7 were significantly affected by storage temperature, juice characteristics and type of microorganism. Malic acid resulted more effective at 35 and 20ºC than at 5ºC in all studied fruit juices. *E. coli* O157:H7 was more resistant to malic acid than *S.* Enteritidis and *L. monocytogenes*. Apple, pear and melon juices without malic acid were inhibitory to *E. coli* O157:H7, *S.* Enteritidis and *L. monocytogenes* at 5ºC, whereas, MBCs of 1.5% of malic acid in apple and pear juices, and 2% in melon juice at 5ºC were needed to reduce *E. coli* O157:H7, being those concentrations higher than those required to reduce *S.* Enteritidis and *L. monocytogenes* in those fruit juices. In addition, concentrations of 2, 2.5 and 2.5% of malic acid were required to inactivate the three pathogens by more than 5 Log cycles after 24h of storage at 5ºC. Transmission electron microscopy showed that malic acid produced damages in cell cytoplasm of pathogens without apparent changes in the cell membrane.

Key words: malic acid; pathogens; fruit juices

1-INTRODUCTION

The consumption of unpasteurized fruit juices defined as the product obtained by pressing or squeezing of the fruits (Harris *et al*., 2003) has increased in the last years presumably due, in part, to their characteristics of freshness, high vitamins content, low calorie contribution, and an active promotion of fruits and their derivatives as important components of a healthy diet. However, foodborne disease outbreaks caused by *Escherichia coli* O157:H7 and different serovars of *Salmonella* have been associated to unpasteurized fruit juices (CDC 2007; Harris *et al*., 2003) demonstrating that those products can serve as harbor of pathogenic microorganisms. In addition, incidence or survival/growth of *L. monocytogenes, L. innocua*, *Salmonella* serovars and *E. coli* O157:H7 in fruit juices and apple cider has been demonstrated (Miller and Kaspar, 1994; Harris *et al*., 2003; Ceylan, Fung & Sabah, 2004; Ingham, Schoeller, Engel, 2006; Raybaudi-Massilia, Mosqueda-Melgar & Martín-Belloso, 2006). In response to the high outbreaks number caused by those pathogenic microorganisms by consumption of fresh products, the Regulatory Organizations have recommended the use of good cleaning and sanitation practices (Garcia, Henderson, Fabri & Oke, 2006) as well as the application of a hazard analysis and critical control point program for juices production (McLellan & Splittstoesser, 1996). Likewise, the Food and Drug Administration has established regulations for juice manufacturing, indicating that treatments for commercial preparation of fresh juices should be capable of reducing pathogenic loads by a minimum of 5.0 log (USFDA, 2002; Derrickson-Tharrington, Kendall, & Sofos, 2005).

The use of organic acids is considered as a good alternative in the fruit processing industry because of their natural origin and preservative, antioxidant, flavoring and acidifying properties as well as by their low cost. However, some important aspects such as kind of juice, characteristics of the spoilage or pathogenic flora and characteristics of the acid must be considered before selecting an acid as antimicrobial agent for fruit juices. Different studies in vitro about the pH effect on *L. monocytogenes*, *Salmonella* and *E. coli* O157:H7 have shown that the inhibitory or bactericidal effect depends of the characteristics of the acid used to adjust the medium pH (Parish and Higgins, 1989; Buchanan and Klawitter, 1990; Chung and Goepfert, 1970; Glass, Loeffelholz, Ford & Doyle, 1992). Thus, variations in effectiveness among acids depend on their molecular structure, size and pKa (Chung and Goepfert, 1970, Parish and Higings, 1989, Eswaranandam, Hettiarachchy & Johnson, 2004). In addition, the acid-

tolerance of microorganisms could also affect the effectiveness of organic acids as antimicrobial agents. Hence, studies that show the minimal inhibitory and bactericidal concentrations of specific organic acids against those pathogenic microorganisms in fruit juices may be of great interest for the industry. Malic acid is a relatively new and emerging acidulant preservative, with the potential to impart excellent sensory property to the fruit products (Raju and Bawa, 2006) that is generally recognized as safe (GRAS) by the FDA (USFDA, 2006) which could be considered as not lipophilic according to its low partition coefficient -1.26 Log octanol/water (Leo, Hansch & Elkins, 1971), thus being its mode of antimicrobial action mainly attributed to the reduction of the medium pH (Beuchat and Golden, 1989). However, some authors have indicated that its low molecular size can permit a free diffusion across of the cell membrane causing significant damages in the cell cytoplasm (Eswaranandam *et al*., 2004). Therefore, a better understanding about the mode of antimicrobial action of malic acid is still necessary.

The objective of the present study was to determine the minimal inhibitory (MIC) and minimal bactericidal (MBC) concentrations of malic acid against *L. monocytogenes*, *S.* Enteritidis and *E. coli* O157:H7 in apple, pear and melon juices stored at 5, 20 and 35ºC as well as to evaluate the mode of antimicrobial action of the malic acid.

2- MATERIALS AND METHODS

2.1.- Fruits and juices preparation

"Fuji" apples (*Malus domestica* Borkh), "Flor de invierno" pears (*Pyrus communis* L.) and "Piel de sapo" melons (*Cucumis melo* L.) at commercial ripeness were purchased in a supermarket of Lleida (Spain) for preparing fruit juices. Each fruit was washed, peeled, cut into pieces and blended using an Ufesa blender (Model BP 4512; Vitoria, Spain). Fruit juices obtained were then centrifuged at 12500 rpm for 15 min. at 4º C in an Avanti™ J-25 Centrifuge (Beckman Instrument, Inc.; USA). Each of supernatant juice was filtered, bottled and autoclaved in a Presoclave 75 (J.P. Selecta, S.A; Barcelona, Spain) at 121º C for 15 min.

2.2- Addition of malic acid to fruit juices

D-L -malic acid, extra pure (Scharlau Chemie S. A., Barcelona, Spain), was added to one hundred milliliters of sterile apple, pear and melon juices individually bottled into 150 ml sterilized polypropylene containers with polyethylene screw-cap (Deltalab, Barcelona, Spain) under a horizontal

laminar air flow cabin (Telstar, S.A., Barcelona, Spain) in aseptic conditions to obtain fruit juices at final malic acid concentrations of 0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.5, 2.0 and 2.5%. A pair of containers of each fruit juice and malic acid concentration was prepared. Experiment was carried out twice.

2.3- Cultures and inoculation process

Listeria monocytogenes 1.131 (CECT 932) and *Escherichia coli* O157:H7 (CECT 4267) from the Spanish Type Culture Collection of the University of Valencia, Valencia, Spain, and *Salmonella* Enteritidis 1.82 (NCTC 9001) from the National Collection of Type Culture of the Central Public Health Laboratory; London, UK, were maintained in tryptone soy agar (TSA) (Biokar Diagnostics. Beauvais, France) slants at 5ºC until its use. Stock cultures of *L. monocytogenes* and *E. coli* O157:H7 were grown in tryptone soy broth (TSB) (Biokar Diagnostics) with 0.6% yeast extract (YE) (Biokar Diagnostics); whereas, *S.* Enteritidis was cultured in TSB. *E. coli* O157:H7 and *S.* Enteritidis were incubated at 37ºC with continuous agitation for 11 hours at 120 rpm, while *L. monocytogenes* was incubated at 35ºC with continuous shaking for 15 hours at 200 rpm to obtain cells in early stationary growth phase. The maximum growth for *L. monocytogenes*, *S.* Enteritidis and *E. coli* O157:H7 was 10^9 colonies forming units/milliliter (CFU/ml). Concentrations were then adjusted to 10^8 CFU/ml using saline peptone water (0.1% peptone plus 0.85% NaCl, Scharlau Chemie, S.A. Barcelona, Spain). An aliquot of 1ml of bacterial suspension (*L. monocytogenes*, *S.* Enteritidis or *E. coli* O157:H7) at approximately 10^8 CFU/ml was individually added to each fruit juice sample containing malic acid in different concentrations. A control of each juice (apple, pear and melon) without malic acid was also inoculated.

2.4- Determination of Minimal inhibitory (MIC) and minimal bactericidal (MBC) concentrations

MICs and MBCs of malic acid against *L. monocytogenes*, *S.* Enteritidis and *E. coli* O157:H7 were determined by the broth dilution method reported by Davidson and Parish (1989). For that, apple, pear and melon juices with or without malic acid added and individually inoculated with *L. monocytogenes*, *S.* Enteritidis and *E. coli* O157:H7 were incubated at 5ºC (temperature normally used for their preservation) for 120h and, at 20 and 35ºC during 24h to simulate abuse temperatures. Afterwards, an aliquot of 1ml of those incubated fruit juices was poured into TSA plates to check bacterial survival, whereas a 500 µl aliquot was poured into TSB medium (4.5 ml) to check injured or death cells. Those plates and tubes were

incubated at 35ºC for 24h. The MIC was considered as the lowest concentration to maintain or reduce ≤ 1 Log CFU/ml the inoculum level, whereas, the MBC was considered as the lowest concentration where a reduction > 1 Log CFU/ml of the inoculated population was observed. In addition, the necessary minimum concentration to inactivate more than 5 Log CFU/ml of each microorganism was established after examination of the plates and tubes.

2.5- pH Determination

The pH of apple, pear and melon juices with different concentrations of malic acid was determined (Table 1) using a Microprocessor pH meter Hanna Instruments PH210 (Vernon Hills, USA).

Table 1. pH values of apple, pear and melon juices with different concentrations of malic acid

Acid concentration (%)	pH*		
	Apple	Pear	Melon
0	3.94 ± 0.01	4.60 ± 0.03	5.45 ± 0.21
0.2	3.57 ± 0.01	3.73 ± 0.01	4.31 ± 0.04
0.4	3.31 ± 0.02	3.45 ± 0.04	3.84 ± 0.03
0.6	3.13 ± 0.03	3.25 ± 0.02	3.62 ± 0.02
0.8	3.06 ± 0.01	3.20 ± 0.15	3.47 ± 0.01
1.0	2.97 ± 0.02	2.99 ± 0.01	3.32 ± 0.04
1.5	2.79 ± 0.04	2.81 ± 0.03	3.13 ± 0.03
2.0	2.68 ± 0.04	2.65 ± 0.01	3.03 ± 0.01
2.5	2.61 ± 0.01	2.51 ± 0.02	2.91 ± 0.01

***** Means ± standard deviation obtained in two determinations, each one in duplicated (n = 4)

2.6- Transmission Electron Microscopy (TEM)

Cells of *L. monocytogenes*, *S.* Enteritidis and *E. coli* O157:H7 were cultured in TSB medium as in section 2.3, fruit juices (melon, pear and apple juices) and fruit juices with malic acid. Afterwards, they were fixed in glutaraldehyde (2.5 % in 0.1 M phosphate buffer, pH 7.4) for 1 h, rinsed three times for 10 min with 0.1 M phosphate buffer (pH 7.4) and post fixed with 1 % osmium tetraoxide for 2 h a 4°C. After fixation, the cells were rinsed three times for 10 min with 0.1 M phosphate buffer (pH 7.4) and then dehydrated using 30, 50, 70, and 95 % acetone sequentially for 15 min each. Next, the cells were dehydrated three times for 30 min with 100 % acetone. After dehydration, the cells were treated with propylene oxide twice for 10

min a 4°C. The cells were sequentially infiltrated with a mixture of propylene oxide: Durcupan's ACM Epoxy Resin (3:1, 1:1, and 1:3) for 45 min. Polymerization of the resin to form specimen blocks was performed in an oven at 60°C for 72 h. The specimen blocks were hand trimmed with a razor blade and sectioned with a diamond knife in a Reichert Ultracut R ultramicrotome (Leica, Wetzler, Germany). Thin sections (70 to 80 nm) were placed on 300-mesh copper grids. The sections were stained for 15-20 min in uranyl: ethyl alcohol (1:1), after washed 3 times for 2 min and then incubated in a drop of Reynold's lead citrate and examined using an EM 910 Zeiss transmission electron microscope, Germany.

2.7- Statistical analysis

Statistical analysis of the microbial counts was performed individually for each microorganism using the Statgraphics plus v.5.1 software. A multifactor analysis of variance with posterior multiple range test was used to find significant differences ($p < 0.05$) within kinds of juice (apple, pear and melon), range of concentrations of malic acid (levels: 0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.5, 2.0 and 2.5 %) and storage temperatures (5, 20 and 35ºC) evaluated.

3- RESULTS AND DISCUSSION

3.1-Minimal inhibitory concentration (MIC)

Growth of *L. monocytogenes*, *S.* Enteritids and *E. coli* O157:H7 in apple (pH 3.94), pear (pH 4.60) and melon (pH 5.45) juices without addition of malic acid stored at 5 ºC for 120 h was not observed (Tables 2-4). Therefore, the establishment of MIC of malic acid in these cases was not necessary. Similar results were reported by Ceylan *et al*. (2004) who demonstrated that *E. coli* O157:H7 population showed negligible changes in apple juice (pH 3.75) stored at 8ºC for 14 days. Likewise, Miller and Kaspar (1994) indicated that *E. coli* O157:H7 population was unchanged in apple cider (3.7-4.1) stored at 4ºC for 14 days. Yuste and Fung (2002) also reported survival but not growth of *L. monocytogenes* in apple juice (pH 3.7) at 5ºC.

Apple and pear juices stored at 20 and 35ºC did not show growth of *L. monocytogenes*, *S.* Enteritidis and *E. coli* O157:H7 populations either after 24h (Tables 5-7). A similar behavior was reported by Raybaudi-Massilia *et al*. (2006) on *L. innocua, S.* Enteritidis and *E. coli* inoculated in apple and pear juices stored at 35ºC. Likewise, Yuste and Fung (2002) did not observe

Table 2. Effect of malic acid concentration and storage time on *Listeria monocytogenes* inoculated in apple, pear and melon juices stored at 5ºC

Storage time (h)	Malic acid (%)	Survival population in juice (Log_{10} CFU/ml)*		
		Apple	Pear	Melon
0	**0**	7.03 ± 0.04 Aaα	6.75 ± 0.24 Aaβ	6.880 ± 0.007 Aaδ
	0.2	6.96 ± 0.05 Aabα	6.68 ± 0.28 Aaβ	6.54 ± 0.09 Abδ
	0.4	6.924 ± 0.029 Abα	6.6 ± 0.4 Aaβ	6.45 ± 0.08 Abcδ
	0.6	6.884 ± 0.012 Abα	6.44 ± 0.06 Aaβ	6.42 ± 0.07 Abcδ
	0.8	6.112 ± 0.017 Acα	6.412 ± 0.005 Aaβ	6.27 ± 0.09 Acdδ
	1.0	5.42 ± 0.08 Adα	5.50 ± 0.17 Abβ	6.12 ± 0.06 Adδ
	1.5	< 1.0 Aeα	< 1.0 Acβ	2.59 ± 0.16 Aeδ
	2.0	< 1.0 Aeα	< 1.0 Acβ	< 1.0 Afδ
	2.5	< 1.0 Aeα	< 1.0 Acβ	< 1.0 Afδ
24	**0**	6.991 ± 0.025 Baα	6.6 ± 0.4 Baβ	6.79 ± 0.08 Baδ
	0.2	5.57 ± 0.17 Baα	6.5 ± 0.3 Baβ	6.74 ± 0.06 Baδ
	0.4	3.4 ± 0.3 Baα	4.81 ± 0.05 Bbβ	6.60 ± 0.22 Babδ
	0.6	< 1.0 Bbα	1.89 ± 0.16 Bcβ	6.59 ± 0.16 Babcδ
	0.8	< 1.0 Bbα	< 1.0 Bdβ	2.40 ± 0.11 Bbcδ
	1.0	< 1.0 Bbα	< 1.0 Bdβ	< 1.0 Bcδ
	1.5	< 1.0 Bbα	< 1.0 Bdβ	< 1.0 Bcδ
	2.0	< 1.0 Bbα	< 1.0 Bdβ	< 1.0 Bcδ
	2.5	< 1.0 Bbα	< 1.0 Bdβ	< 1.0 Bcδ
48	**0**	6.95 ± 0.04 Caα	6.371 ± 0.013 Caβ	6.76 ± 0.08 Caδ
	0.2	1.78 ± 0.21 Cbα	4.4 ± 0.3 Cbβ	6.75 ± 0.04 Caδ
	0.4	< 1.0 Ccα	1.27 ± 0.22 Ccβ	6.4 ± 0.5 Caδ
	0.6	< 1.0 Ccα	< 1.0 Cdβ	5.47 ± 0.11 Cbδ
	0.8	< 1.0 Ccα	< 1.0 Cdβ	< 1.0 Ccδ
	1.0	< 1.0 Ccα	< 1.0 Cdβ	< 1.0 Ccδ
	1.5	< 1.0 Ccα	< 1.0 Cdβ	< 1.0 Ccδ
	2.0	< 1.0 Ccα	< 1.0 Cdβ	< 1.0 Ccδ
	2.5	< 1.0 Ccα	< 1.0 Cdβ	< 1.0 Ccδ
120	**0**	6.89 ± 0.09 Daα	6.320 ± 0.011 Daβ	6.684 ± 0.022 Daδ
	0.2	< 1.0 Dbα	3.2 ± 0.3Dbβ	6.08 ± 0.26 Dbδ
	0.4	< 1.0 Dbα	1.56 ± 0.13 Dcβ	4.3 ± 0.5 Dcδ
	0.6	< 1.0 Dbα	< 1.0 Ddβ	1.98 ± 0.19 Ddδ
	0.8	< 1.0 Dbα	< 1.0 Ddβ	< 1.0 Deδ
	1.0	< 1.0 Dbα	< 1.0 Ddβ	< 1.0 Deδ
	1.5	< 1.0 Dbα	< 1.0 Ddβ	< 1.0 Deδ
	2.0	< 1.0 Dbα	< 1.0 Ddβ	< 1.0 Deδ
	2.5	< 1.0 Dbα	< 1.0 Ddβ	< 1.0 Deδ

* Means ± standard deviation obtained in two experiments, each one in duplicated (n = 4)

Method detection limit = 1CFU/ml

Different capital letters indicate significant differences ($p<0.05$) among storage times for each juice; different lower-case letters show significant differences ($p<0.05$) among malic acid concentrations for each storage time and juice; different Greek letters demonstrate significant differences ($p<0.05$) among fruit juices for each storage time

Table 3. Effect of malic acid concentration and storage time on *Salmonella* Enteritidis inoculated in apple, pear and melon juices stored at 5ºC

Storage time (h)	Malic acid (%)	Survival population in juice (Log_{10} CFU/ml)*		
		Apple	Pear	Melon
0	0	7.533 ± 0.029 Aaα	6.51 ± 0.05 Aaβ	6.56 ± 0.06 Aaδ
	0.2	7.44 ± 0.09 Aaα	6.54 ± 0.09 Aaβ	6.55 ± 0.10 Aaδ
	0.4	6.97 ± 0.10 Abα	6.51 ± 0.10 Aaβ	6.40 ± 0.14 Aaδ
	0.6	6.79 ± 0.12 Acα	6.34 ± 0.10 Abcβ	6.38 ± 0.03 Aaδ
	0.8	6.62 ± 0.03 Acα	6.25 ± 0.07 Aabβ	6.41 ± 0.09 Aaδ
	1.0	6.32 ± 0.12 Adα	6.22 ± 0.10 Acβ	6.38 ± 0.13 Aaδ
	1.5	5.90 ± 0.05 Aeα	5.093 ± 0.020 Adβ	5.98 ± 0.03 Abδ
	2.0	< 1.0 Afα	< 1.0 Aeβ	< 1.0 Acδ
	2.5	< 1.0 Afα	< 1.0 Aeβ	< 1.0 Acδ
24	0	7.52 ± 0.05 Baα	6.29 ± 0.10 Baβ	6.55 ± 0.05 Babδ
	0.2	7.21 ± 0.13 Bbα	6.19 ± 0.21 Baβ	6.531 ± 0.016 Baδ
	0.4	6.98 ± 0.03 Bcα	6.18 ± 0.26 Baβ	6.50 ± 0.06 Babδ
	0.6	6.92 ± 0.11 Bcα	5.98 ± 0.03 Baβ	6.385 ± 0.019 Bbδ
	0.8	4.33 ± 0.21 Bdα	5.2 ± 0.3 Bbβ	6.15 ± 0.21 Bcδ
	1.0	< 1.0 Beα	2.86 ± 0.07 Bcβ	4.63 ± 0.10 Bdδ
	1.5	< 1.0 Beα	< 1.0 Bdβ	< 1.0 Beδ
	2.0	< 1.0 Beα	< 1.0 Bdβ	< 1.0 Beδ
	2.5	< 1.0 Beα	< 1.0 Bdβ	< 1.0 Beδ
48	0	7.399 ± 0.025 Caα	6.28 ± 0.08 Caβ	6.545 ± 0.008 Caδ
	0.2	7.38 ± 0.17 Caα	6.23 ± 0.16 Caβ	6.54 ± 0.03 Caδ
	0.4	7.2 ± 0.3 Caα	6.15 ± 0.21 Cbβ	6.53 ± 0.03 Caδ
	0.6	5.285 ± 0.010 Cbα	5.656 ± 0.003 Ccβ	6.51 ± 0.16 Caδ
	0.8	< 1.0 Ccα	2.86 ± 0.06 Cdβ	5.36 ± 0.14 Cbδ
	1.0	< 1.0 Ccα	< 1.0 Ceβ	3.30 ± 0.05 Ccδ
	1.5	< 1.0 Ccα	< 1.0 Ceβ	< 1.0 Cdδ
	2.0	< 1.0 Ccα	< 1.0 Ceβ	< 1.0 Cdδ
	2.5	< 1.0 Ccα	< 1.0 Ceβ	< 1.0 Cdδ
120	0	7.15 ± 0.21 Daα	6.216 ± 0.005 Daβ	6.46 ± 0.11 Daδ
	0.2	5.60 ± 0.09 Dbα	6. 2 ± 0.4 Dbβ	6.45 ± 0.01 Daδ
	0.4	3.43 ± 0.16 Dcα	6.09 ± 0.25 Dbβ	6.41 ± 0.06 Dabδ
	0.6	< 1.0 Ddα	3.87 ± 0.08 Dcβ	6.29 ± 0.10 Dbδ
	0.8	< 1.0 Ddα	< 1.0 D dβ	4.76 ± 0.08 Dcδ
	1.0	< 1.0 Ddα	< 1.0 Ddβ	2.59 ± 0.09 Ddδ
	1.5	< 1.0 Ddα	< 1.0 Ddβ	< 1.0 Deδ
	2.0	< 1.0 D dα	< 1.0 Ddβ	< 1.0 Deδ
	2.5	< 1.0 Ddα	< 1.0 Ddβ	< 1.0 Deδ

* Means ± standard deviation obtained in two experiments, each one in duplicated (n = 4)

Method detection limit = 1CFU/ml

Different capital letters indicate significant differences ($p<0.05$) among storage times for each juice; different lower-case letters show significant differences ($p<0.05$) among malic acid concentrations for each storage time and juice; different Greek letters demonstrate significant differences ($p<0.05$) among fruit juices for each storage time

Table 4. Effect of malic acid concentration and storage time on *Escherichia coli* O157:H7 inoculated in apple, pear and melon juices stored at 5ºC

Storage time (h)	Malic acid (%)	Survival population in juice (Log_{10} CFU/ml)*		
		Apple	Pear	Melon
0	**0**	6.90 ± 0.06 Aaα	6.88 ± 0.03 Aabβ	6.89 ± 0.07 Aaδ
	0.2	6.891 ± 0.017 Aaα	6.87 ± 0.03 Abβ	6.85 ± 0.09 Aaδ
	0.4	6.893 ± 0.015 Aaα	6.80 ± 0.05 Abβ	6.79 ± 0.20 Aaδ
	0.6	6.88 ± 0.03 Aaα	6.834 ± 0.016 Aabβ	6.73 ± 0.24 Aaδ
	0.8	6.913 ± 0.017 Aaα	6.81 ± 0.03 Aabβ	6.736 ± 0.010 Aabδ
	1.0	6.911 ± 0.011 Aaα	6.829 ± 0.023 Aabβ	6.502 ± 0.010 Aabδ
	1.5	6.96 ± 0.11 Aaα	6.851 ± 0.009 Aabβ	5.97 ± 0.17 Aabδ
	2.0	6.3 ± 0.5 Abα	6.694 ± 0.019 Acβ	5.83 ± 0.19 Aabδ
	2.5	3.02 ± 0.21 Acα	5.83 ± 0.03 Adβ	5.703 ± 0.018 Abδ
24	**0**	6.75 ± 0.08 Baα	6.633 ± 0.029 Baβ	6.85 ± 0.08 Baδ
	0.2	6.74 ± 0.06 Baα	6.59 ± 0.16 Baβ	6.82 ± 0.06 Baδ
	0.4	6.68 ± 0.04 Baα	6.523 ± 0.112 Baβ	6.79 ± 0.08 Baδ
	0.6	6.712 ± 0.018 Baα	6.39 ± 0.12 Baβ	6.70 ± 0.06 Baδ
	0.8	6.45 ± 0.21 Babα	6.2 ± 0.3 Babβ	6.72 ± 0.17 Baδ
	1.0	6.3 ± 0.4 Bbα	5.95 ± 0.07 Bbβ	6.4 ± 0.6 Baδ
	1.5	3.54 ± 0.09 Bcα	3.64 ± 0.23 Bcβ	5.4 ± 0.4 Bbδ
	2.0	< 1.0 Bdα	1.2 ± 0.3 Bdβ	3.2 ± 0.4 Bcδ
	2.5	< 1.0 Bdα	< 1.0 Beβ	< 1.0 Bdδ
48	**0**	6.716 ± 0.023 Caα	6.56 ± 0.12 Caβ	6.80 ± 0.08 Caδ
	0.2	6.62 ± 0.22 Caα	6.53 ± 0.10 Caβ	6.75 ± 0.04 Caδ
	0.4	6.1 ± 0.4 Cbα	6.53 ± 0.11 Caβ	6.77 ± 0.04 Caδ
	0.6	5.7 ± 0.3 Cbα	6.41 ± 0.15 Caβ	6.737 ± 0.016 Caδ
	0.8	5.1 ± 0.3 Ccα	6.60 ± 0.08 Caβ	6.73 ± 0.03 Caδ
	1.0	3.641 ± 0.018 dCα	3.68 ± 0.14 Cbβ	6.31 ± 0.05 Cbδ
	1.5	1.380 ± 0.026 Ceα	1.44 ± 0.19 Ccβ	4.72 ± 0.11 Ccδ
	2.0	< 1.0 Cfα	< 1.0 Cdβ	< 1.0 Cdδ
	2.5	< 1.0 Cfα	< 1.0 Cdβ	< 1.0 Cdδ
120	**0**	6.54 ± 0.09 Daα	6.20 ± 0.20 Daβ	6.78 ± 0.05 Daδ
	0.2	6.51 ± 0.20 Daα	6.10 ± 0.07 Dabβ	6.741 ± 0.010 Dabδ
	0.4	6.25 ± 0.18 Daα	6.11 ± 0.22 Dabβ	6.72 ± 0.17 Dabδ
	0.6	5.3 ± 0.4 Dbα	5.92 ± 0.08 Dbβ	6.61 ± 0.10 Dbδ
	0.8	2.84 ± 0.05 Dcα	4.82 ± 0.04 Dcβ	6.037 ± 0.025 Dcδ
	1.0	2.16 ± 0.16 Ddα	2.77 ± 0.11 Ddβ	2.80 ± 0.28 Ddδ
	1.5	< 1.0 Deα	< 1.0 Deβ	< 1.0 Deδ
	2.0	< 1.0 Deα	< 1.0 Deβ	< 1.0 Deδ
	2.5	< 1.0 Deα	< 1.0 Deβ	< 1.0 Deδ

* Means ± standard deviation obtained in two experiments, each one in duplicated (n = 4)

Method detection limit = 1CFU/ml

Different capital letters indicate significant differences ($p<0.05$) among storage times for each juice; different lower-case letters show significant differences ($p<0.05$) among malic acid concentrations for each storage time and juice; different Greek letters demonstrate significant differences ($p<0.05$) among fruit juices for each storage time

growth of *L. monocytogenes* in apple juice (pH 3.7) stored at 20ºC. In addition, survival but not growth of *E. coli* O157:H7 in apple cider (pH 3.6 to 4.0) from 2 to 3 days at 25ºC as well as in apple juice (pH 3.75) by more than 3 days at 25ºC were reported by Zhao, Doyle, & Besser (1993) and Ceylan *et al*. (2004), respectively. By the contrary, melon juice stored at 20 and 35ºC did not inhibit the growth of *L. monocytogenes*, *S.* Enteritidis and *E. coli* O157:H7, since those populations significantly increased after 24h (Tables 5-7). The microbial growth in melon juice is a direct consequence of its initial pH (Table 1), which is higher than the minimal pH of growing reported for *L. monocytogenes* (4.4), *S.* Enteritidis (3.99) and *E. coli* O157:H7 (4.0 to 4.5) in food (Lou and Yousef ,1999; D'Aoust, Maurer & Bailey, 2001; Meng, Doyle, Zhao & Zhao, 2001). Growth of *L. innocua*, *S.* Enteritidis and *E. coli* in melon juice at pH 5.91 stored at 35ºC was also observed by Raybaudi-Massilia *et al*. (2006).

Table 5. Effect of malic acid concentration and storage time on *Listeria monocytogenes* inoculated in apple, pear and melon juices stored at 20 and 35ºC

Time (h)	Malic acid (%)	Survival population in Juice (Log_{10} CFU/ml)*					
		Apple		Pear		Melon	
		20ºC	35ºC	20ºC	35ºC	20ºC	35ºC
0	0	7.36 ± 0.05 Aaα		6.61 ± 0.12 Aaβ		6.633 ± 0.020 Aaδ	
	0.2	7.07 ± 0.04 Aaα		6.36 ± 0.18 Aaβ		6.57 ± 0.07 Aaδ	
	0.4	7.00 ± 0.05 Aaα		6.6 ± 0.3 Aaβ		6.46 ± 0.28 Aabδ	
	0.6	6.67 ± 0.04 Abα		6.43 ± 0.26 Aaβ		6.32 ± 0.11 Abcδ	
	0.8	6.37 ± 0.10 Acα		5.75 ± 0.09 Abβ		6.17 ± 0.10 Acδ	
	1.0	4.91 ± 0.15 Adα		5.06 ± 0.15 Acβ		6.08 ± 0.09 Adδ	
	1.5	< 1.0 Aeα		< 1.0 Adβ		2.81 ± 0.23 Aeδ	
	2.0	< 1.0 Aeα		< 1.0 Adβ		< 1.0 Afδ	
	2.5	< 1.0 Aeα		< 1.0 Adβ		< 1.0 Afδ	
24	0	6.44 ± 0.08 Baα	6.780± 0.013 Baα	6.44 ±0.18 Baβ	6.61± 0.05 Baβ	8.54± 0.03 Baδ	8.7± 0.1 Baδ
	0.2	2.60 ± 0.18 Bbα	1.34± 0.06 Bbα	6.2 ±0.3 Baβ	6.20± 0.28 Bbβ	6.44± 0.08 Bbδ	6.48± 0.20 Bbδ
	0.4	< 1.0 Bcα	< 1.0 Bcα	2.13 ±0.07 Bbβ	1.87± 0.13 Bcβ	6.30± 0.03 Bcδ	6.30± 0.06 Bbδ
	0.6	< 1.0 Bcα	< 1.0 Bcα	< 1.0 Bcβ	< 1.0 Bdβ	5.30± 0.04 Bdδ	< 1.0 Bcδ
	0.8	< 1.0 Bcα	< 1.0 Bcα	< 1.0 Bcβ	< 1.0 Bdβ	< 1.0 Beδ	< 1.0 Bcδ
	1.0	< 1.0 Bcα	< 1.0 Bcα	< 1.0 Bcβ	< 1.0 Bdβ	< 1.0 Beδ	< 1.0 Bcδ
	1.5	< 1.0 Bcα	< 1.0 Bcα	< 1.0 Bcβ	< 1.0 Bdβ	< 1.0 Beδ	< 1.0 Bcδ
	2.0	< 1.0 Bcα	< 1.0 Bcα	< 1.0 Bcβ	< 1.0 Bdβ	< 1.0 Beδ	< 1.0 Bcδ
	2.5	< 1.0 Bcα	< 1.0 Bαc	< 1.0 Bcβ	< 1.0 Bdβ	< 1.0 Beδ	< 1.0 Bcδ

* Means ± standard deviation obtained in two experiments, each one in duplicated (n = 4)
Method detection limit = 1CFU/ml
Different capital letters indicate significant differences ($p<0.05$) among storage times for each juice; different lower-case letters show significant differences ($p<0.05$) among malic acid concentrations for each storage time and juice; different Greek letters demonstrate significant differences ($p<0.05$) among fruit juices for each storage time

Table 6. Effect of malic acid concentration and storage time on *Salmonella* Enteritidis inoculated in apple, pear and melon juices stored at 20 and 35ºC

Time (h)	Malic acid (%)	Survival population in Juice (Log_{10} CFU/ml)*					
		Apple		Pear		Melon	
		20ºC	35ºC	20ºC	35ºC	20ºC	35ºC
0	0	7.548 ± 0.010 Aaα		6.25 ± 0.05 Aaβ		6.220 ± 0.018 Aaδ	
	0.2	7.37 ± 0.19 Aabα		6.12 ± 0.11 Aaβ		6.210 ± 0.023 Aaδ	
	0.4	7.50 ± 0.03 Aaα		6.01 ± 0.06 Abβ		6.00 ± 0.08 Abδ	
	0.6	7.422 ± 0.013 Abα		5.953 ± 0.029 Abβ		6.08 ± 0.05 Abδ	
	0.8	7.22 ± 0.05 Acα		5.81 ± 0.20 Abcβ		5.91± 0.12 Abcδ	
	1.0	6.79 ± 0.02 Adα		5.72 ± 0.16 Acβ		5.88 ± 0.09 Acδ	
	1.5	3.895 ± 0.03 Aeα		4.5 ± 0.3 Adβ		5.584 ± 0.027 Adδ	
	2.0	< 1.0 Afα		< 1.0 Aeβ		< 1.0 Aeδ	
	2.5	< 1.0 Afα		< 1.0 Aeβ		< 1.0 Aeδ	
24	0	6.98± 0.17 Baα	6.735 ± 0.018 Baα	5.529 ±0.095 Baβ	5.653± 0.027 Baβ	8.18±0.10 Baδ	8.744± 0.006 Baδ
	0.2	6.10± 0.03 Bbα	5.67 ± 0.01 Bbα	5.39 ±0.17 Baβ	5.71± 0.06 Baβ	6.00±0.11 Bbδ	6.171± 0.013 Bbδ
	0.4	5.3± 0.3 Bcα	3.285 ± 0.022 Bcα	5.15 ±0.07 Bbβ	4.5± 0.1 Bbβ	5.26±0.12 Bcδ	5.71± 0.05 Bcδ
	0.6	2.92± 0.09 Bdα	2.04 ± 0.06 Bdα	3.37 ±0.22 Bcβ	1.00± 0.10 Bcβ	3.68±0.14 Bdδ	2.40± 0.09 Bdδ
	0.8	< 1.0 Beα	< 1.0 Beα	< 1.0 Bdβ	< 1.0 Bdβ	< 1.0 Beδ	< 1.0 Beδ
	1.0	< 1.0 Beα	< 1.0 Beα	< 1.0 Bdβ	< 1.0 Bdβ	< 1.0 Beδ	< 1.0 Beδ
	1.5	< 1.0 Beα	< 1.0 Beα	< 1.0 Bdβ	< 1.0 Bdβ	< 1.0 Beδ	< 1.0 Beδ
	2.0	< 1.0 Beα	< 1.0 Beα	< 1.0 Bdβ	< 1.0 Bdβ	< 1.0 Beδ	< 1.0 Beδ
	2.5	< 1.0 Beα	< 1.0 Beα	< 1.0 Bdβ	< 1.0 Bdβ	< 1.0 Beδ	< 1.0 Beδ

* Means ± standard deviation obtained in two experiments, each one in duplicated (n = 4)
Method detection limit = 1CFU/ml
Different capital letters indicate significant differences ($p<0.05$) among storage times for each juice; different lower-case letters show significant differences ($p<0.05$) among malic acid concentrations for each storage time and juice; different Greek letters demonstrate significant differences ($p<0.05$) among fruit juices for each storage time

In this study, MICs of malic acid against *L. monocytogenes*, *S.* Enteritidis and *E. coli* O157:H7 in melon juice at 20 and 35ºC were different, being *S.* Enteritidis and *L. monocytogenes* more sensible than *E. coli* O157:H7, since a concentration of 0.2% of malic acid was enough to inhibit the growth of *S.* Enteritidis and *L. monocytogenes* after 24 h of storage, whereas a concentration of 0.4% was necessary to inhibit the *E. coli* O157:H7 growth under the same conditions (Tables 5-7).

3.2- Minimal bactericidal concentration (MBC)

Malic acid resulted to be effective for reducing and inactivating until undetectable levels *L. monocytogenes, S.* Enteritidis and *E. coli* O157:H7 populations in apple, pear and melon juices, with different bactericidal activity of the acid depending on storage temperature, kind of juice, malic acid concentration, microorganism type (Gram positive or Gram negative) and acid tolerance of each microorganism.

Table 7. Effect of malic acid concentration and storage time on *Escherichia coli* O157:H7 inoculated in apple, pear and melon juices stored at 20 and 35ºC

Time (h)	Malic acid (%)	Survival population in juice (Log_{10} CFU/ml)*					
		Apple		Pear		Melon	
		20ºC	35ºC	20ºC	35ºC	20ºC	35ºC
0	0	6.568 ± 0.017 Aaα		6.47 ± 0.04 Aaβ		6.554 ± 0.021 Aaδ	
	0.2	6.57 ± 0.16 Aaα		6.47 ± 009 Aaβ		6.55 ± 0.08 Aaδ	
	0.4	6.57 ± 0.18 Aaα		6.404 ± 0.028 Aaβ		6.545 ± 0.14 Aaδ	
	0.6	6.67 ± 0.08 Aaα		6.43 ± 0.10 Aaβ		6.51 ± 0.16 Aaδ	
	0.8	6.68 ± 0.04 Aaα		6.41 ± 0.08 Aaβ		6.50 ± 0.07 Aaδ	
	1.0	6.52 ± 0.12 Aaα		6.429 ± 0.017 Aaβ		6.496 ± 0.021 Aaδ	
	1.5	6.614 ± 0.004 Aaα		6.45± 0.05 Aaβ		6.501 ± 0.027 Aaδ	
	2.0	6.62 ± 0.05 Aaα		6.29 ± 0.10 Abβ		6.47 ± 0.19 Aaδ	
	2.5	2.94 ± 0.14 Abα		5.83 ± 0.21 Acβ		6.3 ± 0.3 Abδ	
24	0	6.57 ± 0.03 Baα	6.74 ± 0.01 Baα	6.21 ± 0.03 Baβ	5.958 ± 0.002 Baβ	8.59 ± 0.24 Baδ	8.648 ± 0.007 Baδ
	0.2	6.587 ± 0.010 Baα	5.72 ± 0.03 Bbα	6.15 ± 0.21 Babβ	5.909 ± 0.008 Baβ	6.73 ± 0.07 Bbδ	6.89 ± 0.06 Bbδ
	0.4	6.589 ± 0.006 Baα	5.06 ± 0.03 Bcα	6.07 ± 0.10 Bbβ	5.52 ± 0.07 Bbβ	6.540 ± 0.029 Bcδ	5.562 ± 0.014 Bcδ
	0.6	3.188 ± 0.023 Bbα	3.09 ± 0.02 Bdα	6.30 ± 0.05 Bcβ	4.35 ± 0.07 Bcβ	6.52 ± 0.06 Bcδ	5.11 ± 0.16 Bdδ
	0.8	< 1.0 Bcα	< 1.0 Beα	6.30 ± 0.03 Bcβ	2.618 ± 0.022 Bdβ	6.23 ± 0.09 Bdδ	1.30 ± 0.00 Beδ
	1.0	< 1.0 Bcα	< 1.0 Beα	3.83 ± 0.07 Bdβ	2.52 ± 0.11 Bdβ	6.36 ± 0.08 Beδ	1.36 ± 0.11 Beδ
	1.5	< 1.0 Bcα	< 1.0 Beα	< 1.0 Beβ	< 1.0 Beβ	5.97 ± 0.14 Bfδ	< 1.0 Bfδ
	2.0	< 1.0 Bcα	< 1.0 Beα	< 1.0 Beβ	< 1.0 Beβ	2.13 ± 0.21 Bgδ	< 1.0 Bfδ
	2.5	< 1.0 Bcα	< 1.0 Beα	< 1.0 Beβ	< 1.0 Beβ	< 1.0 Bhδ	< 1.0 Bfδ

* Means ± standard deviation obtained in two experiments, each one in duplicated (n = 4)
Method detection limit = 1CFU/ml
Different capital letters indicate significant differences ($p<0.05$) among storage times for each juice; different lower-case letters show significant differences ($p<0.05$) among malic acid concentrations for each storage time and juice; different Greek letters demonstrate significant differences ($p<0.05$) among fruit juices for each storage time

Bactericidal action of malic acid in fruit juices, where growth of *L. monocytogenes* S. Enteritidis and *E. coli* O157:H7 was not detected, was confirmed after a cells-injured repair step in TSB medium at 35ºC for 24h. Statistical analysis were made independently for each microorganism at 5, 20 and 35ºC, showing significant differences ($p<0.05$) among microorganism counts depending on the malic acid concentration, kind of juice and storage time.

Higher concentrations of malic acid were generally required to reduce in more than 1 Log cycle (MBCs) populations of *L. monocytogenes,* S. Enteritidis and *E. coli* O157:H7 in melon and pear juices than in apple juice (Tables 2-7), indicating that the initial pH of the growth medium is an important factor to consider when organic acid are used to control pathogenic microorganisms in fruit juices. After 24h of incubation at 20 and 35ºC MBCs of 0.2, 0.4 and 0.6 in apple, pear and melon juices, respectively, were required for *L. monocytogenes* and *S.* Enteritidis, whereas MBCs of 0.6, 1 and 2% at 20ºC and 0.4, 0.6 and 0.8 at 35ºC

were needed for *E. coli* O157:H7 in those juices (Tables 5-7). However, at 5ºC apple and pear juices required the same MBCs for each pathogen after 24h of storage, in contrast with melon juice where higher MBCs were needed (Tables 2-4).

On the other hand, storage time had significant influence ($p<0.05$) over the MBCs required to reduce or inactivate *L. monocytogenes* S. Enteritidis and *E. coli* O157:H7, thus decreasing the MBCs and minimal concentration for totally inactivating those microorganisms when storage time increased (Tables 2-7). Hence, concentrations of 1.5, 1.5 and 2% were required to immediately inactivate (t = 0h) *L. monocytogenes* in apple, pear and melon juices, whereas concentrations over 2% and 2.5% were needed to achieve that same effect in *S.* Enteritidis and *E. coli* O157:H7 populations, respectively, in those juices. However, after 24h of storage at 5ºC, lower concentrations of malic acid (0.6, 0.8 and 1%) caused that same inactivation in *L. monocytogenes* in those fruit juices, whereas concentrations of 1, 1.5 and 1.5% for *S.* Enteritidis and 2, 2.5 and 2.5% for *E. coli* O157:H7 were enough to inactivate them (Tables 2-4). At 20 and 35ºC, a similar effect of the storage time over the inactivation of those microorganisms was observed (Tables 5-7).

Significant influence of the storage temperature on the reductions of pathogenic microorganisms in fruit juices was observed. In general, higher concentrations of malic acid were required to reduce *L. monocytogenes* S. Enteritidis and *E. coli* O157:H7 in those fruit juices stored at 5ºC than at 20 and 35ºC (Tables 2-7). Thus, MBCs of 0.4% of malic acid in apple and pear juices and 0,8% in melon juice stored at 5ºC for 24h were required for reducing *L. monocytogenes*, whereas for *S.* Enteritidis (0.8 and 1%) and *E. coli* O157:H7 (1.5 and 2%) higher MBCs in those juices were needed. According to those results Conner and Kotrola, (1995) reported that malic acid at 0.6% added to TSB medium with YE resulted inhibitory but not bactericide for *E. coli* O157:H7 during approximately 35 days at 4ºC. Nevertheless, at 20 and 35ºC MBCs of 0.2, 0.4 and 0.6% for *L. monocytogenes* and *S.* Enteritidis were found in apple, pear and melon juices, respectively, whereas for *E. coli* O157:H7 0.6, 1 and 2% at 20ºC and 0.4, 0.6 and 0.8% at 35ºC were needed in those juices. Likewise, minimal concentrations to reduce in more than 5 cycles those pathogens in fruit juices were also higher at 5ºC than 20 and 35ºC. In general, concentrations of 2% of malic acid in apple juice and 2.5% in pear and melon juices were necessary for inactivate totally *L. monocytogenes* S. Enteritidis and *E. coli* O157:H7 after 24h of incubation at 5ºC (Tables 2-4), whereas lower concentrations of that acid in apple, pear and melon juices were enough to reach that same

effect over those pathogens at 20ºC (0.8, 1.5 and 2.5%) and 35ºC (0.8, 1.5 and 1.5%) after 24h of storage (Tables 5-7). A greater fluidity of the cellular membrane of the microorganisms at high temperatures (> 20ºC) could favor the entry of malic acid to the cell inside. In such sense, Aronsson and Rönner (2001) indicated that the temperature of the medium in which cells are suspended has a significant influence on the membrane fluidity properties. At low temperatures, the phospholipids are closely packed into a rigid gel structure, while at high temperatures they are less ordered and membrane has a liquid-crystalline structure.

According to the results obtained in this study, temperature of 5ºC should be selected as an indicator temperature when organic acids are used as antimicrobial agents in fruit juices, since pathogenic microorganisms were less affected by malic acid at this temperature in comparison with 20 and 35ºC.

E. coli O157:H7 was more resistant to malic acid than *S.* Enteritids and *L. monocytogenes,* since higher concentrations of malic acid were needed to reduce more than 5 Log cycles of this microorganism in apple, pear and melon juices in comparison with *L. monocytogenes* and *S.* Enteritidis. That result is according to those reported by others authors (Miller and Kaspar, 1994; Benjamin and Datta, 1995: Arnold and Kaspar, 1995: Lin, Smith, Chapin, Baik, Bennett & Foster, 1996) who indicated that acid-resistance is a typical characteristic of this microorganism. Lin *et al*. (1996) indicated that three systems are involved in the acid tolerance of *E. coli* O157:H7, including an acid-induced oxidative system, an acid-induced arginine-dependent system, and a glutamate-dependent system. Therefore, *E. coli* O157:H7 should be considered as a target microorganism to evaluate the effectiveness of organic acids in fruit juices.

3.3- Mode of antimicrobial action of malic acid

Owing to the metabolic complexity of the microbial cell, it is very unlikely that a chemical compound may affect a unique site of action (Stratford and Eklund, 2003). Thus, organic acids in food can likely affect a number of systems in the target microorganism. In general, the antimicrobial activity of organic acids have been attributed to pH reduction, depression of internal pH of microbial cell by ionization of undissociated acid molecules, disruption of substrate transport by altering cell membrane permeability or reduction of proton motive force and chelation of metal ions essential for

microbial growth (Doores 1993; Stratford and Eklund 2003; Eswaranandam *et al*., 2004).

Malic acid is an organic acid of low lipid solubility (Leo *et al*., 1971) and consequently its entry into the cell could be limited, since the cell membrane is impermeable to polar compounds (Lücke, 2003). Therefore, a decrease in the medium pH could explain its antimicrobial action as was suggested by Beuchat and Golden (1989). However, some authors have found that effectiveness of the organic acids can vary depending on its molecular weight. Eswaranandam *et al*., (2004) indicated that undissociated smaller molecules of malic (134.09 Dalton) and lactic (90.08 Dalton) acids may entry into the bacterial cells easily and change the internal pH of the microorganism, thus showing higher antimicrobial activity than undissociated larger molecules of citric (192.13 Dalton) and tartaric (150.09 Dalton) acids, which may not entry toward the cell inside effectively.

A diminution of the intracellular pH due to the entry of the malic acid into the cell would explain the mode of action of this acid, since a higher effectiveness of the malic acid against those bacteria in apple juice was observed as a consequence of the initial pH of that juice which is near to the pKa of this acid (3.40) in comparison with those pH values detected in pear and melon juices (Table 1), implicating a lower dissociation of the acid. It is known that the dissociation of organic acids in a media depends directly of their pKa values and that the antibacterial effectiveness of those acids is dependent of undissociated molecules (Davidson 2001).

Membrane structure and acid-tolerancy of the microorganism can also influence on the antimicrobial action of organic acids. In such sense, Brul and Coote (1999), Seltmann and Holst (2002) and Nikaido (1996; 2003) have reported that the resistance mechanisms in Gram negative bacteria are more complicated than those present in Gram positive bacteria, since contain membranes or membrane-like structures of hydrophobic nature that may block the entry of hydrophilic molecules of low molecular mass such as monosaccharides, amino acids, nucleosides and alkali or alkaline ions, which only can pass through of water-filled channels formed by transmembrane proteins (porins) embedded into the lipid bilayer that permit the hydrophilic transport, whereas the Gram positive cell wall contains only a thick peptidoglycan layer and a lipid bilayer.

The higher membrane permeability of Gram positive bacteria can results in both advantages and disadvantages for the bacteria. A higher permeability clearly enhances the range of substances coming into as nutrients and also facilitates the excretion of bigger molecules to the surrounding medium, improving thus, the conditions of bacterial growth and multiplication. However, this higher permeability may also facilitate the penetration of damaging substances that affect the vital processes of bacteria. Thus, the majority of the Gram positive bacteria are more sensitive to antibiotics and other substances than Gram negative bacteria. These facts would explain the greater sensibility of *L. monocytogenes* (Gram positive) to malic acid than *S.* Enteritidis and *E. coli* O157:H7 (Gram negatives) in fruit juices.

Observations of *L. monocytogenes*, *S.* Enteritidis and *E. coli* O157:H7 cells cultivated in TSB medium, fruit juices (melon, pear and apple) and fruit juices with malic acid by Transmission Electron Microscopy (TEM) allowed to elucidate the action mode of malic acid over those pathogenic microorganisms (Fig. 1-3). Significant damages in cell cytoplasm without apparent changes on the cytoplasm membrane were observed for the three microorganisms irrespective to the fruit juice added with malic acid (Fig. 1c, 2c and 3c). In this way, an agglutination of cytoplasmic content was observed in cells cultivated in fruit juices with malic acid as a consequence of a decrease in the intracellular pH. Undissociated malic acid molecules passed across the cell membrane and then were forced to become dissociate due to the neutral pH of the cell cytoplasm. The released protons caused then the decrease of internal pH which was the responsible of the observed damages in the cytoplasmic content.

The results found in this research demonstrated that malic acid passed to the cell inside regardless of the microorganism type Gram positive or Gram negative (Fig. 1c, 2c and 3c). Hence, the diffusion of malic acid into *L. monocytogenes* cell might have occurred through the peptidoglycan layer, whereas in *S.* Enteritidis and *E. coli* O157:H7 might have taken place through the porins.

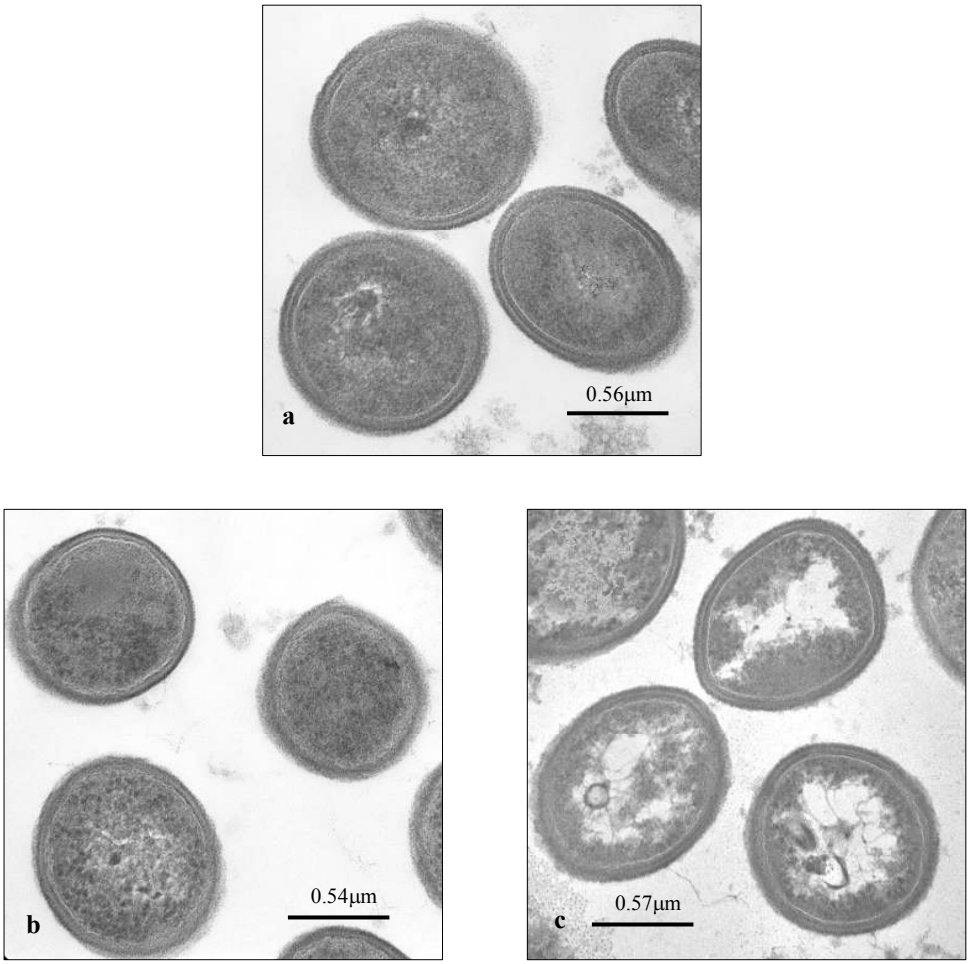

Fig. 1.- Transmission electron microscopy micrographs of (a) *L. monocytogenes* cells from pure culture in TSB plus 0.6% YE grown for 15h at 35ºC and 200 rpm (undamaged cells; *X* 50,000), (b) melon juice (undamaged cells; *X* 50,000) and (c) melon juice with 0.6% malic acid (cells with alteration of cytoplasmic content; *X* 50,000) incubated for 24h at 37ºC.

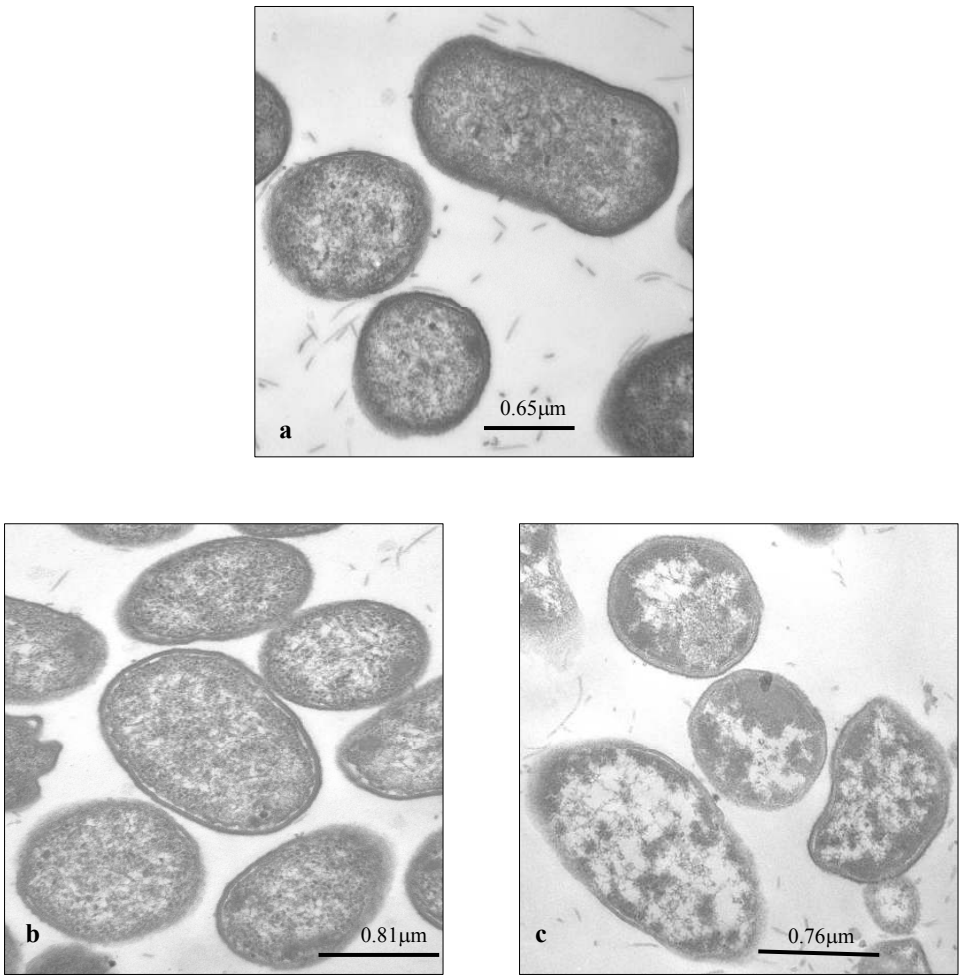

Fig. 2.- Transmission electron microscopy micrographs of (a) *S*. Enteritidis cells from pure culture in TSB grown for 11h at 37ºC and 120 rpm (undamaged cells; *X* 31,500), (b) pear juice (undamaged cells; *X* 31,500) and (c) pear juice with 0.6% malic acid (cells with alteration of cytoplasmic content; *X* 31,500) incubated for 24h at 37ºC.

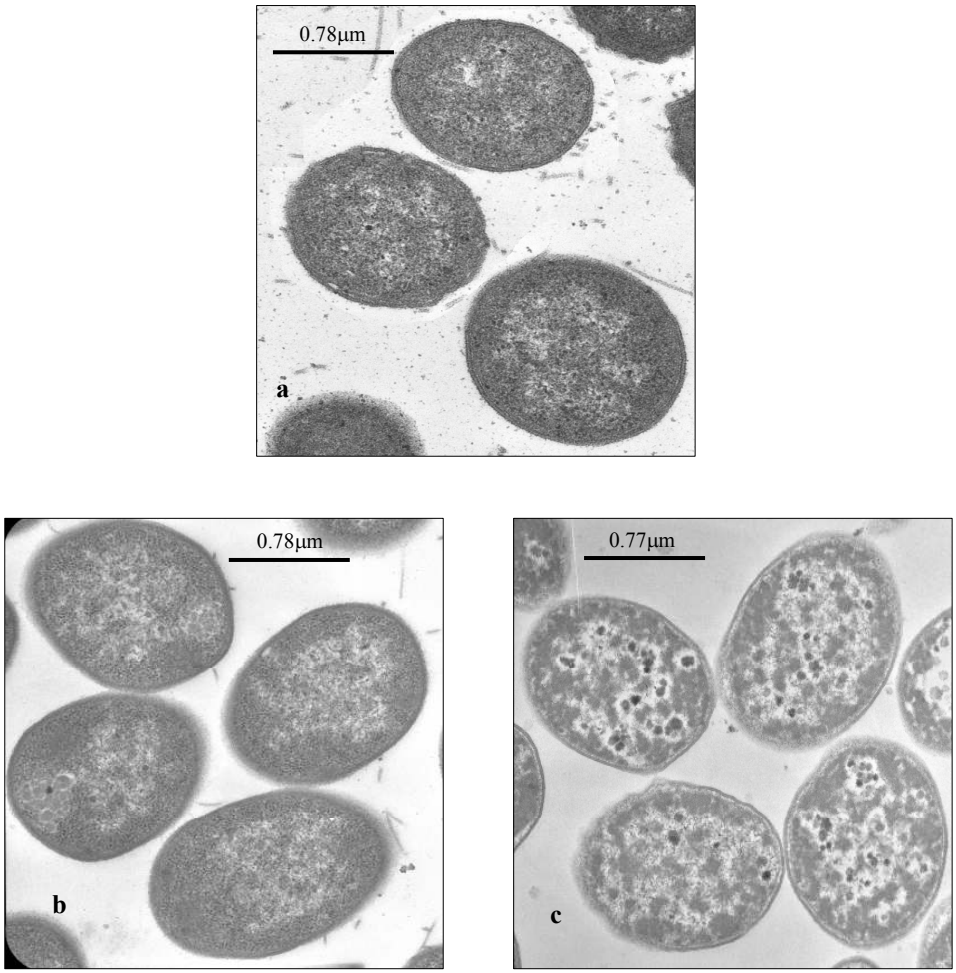

Fig. 3.- Transmission electron microscopy micrographs of (a) *E. coli* O157:H7 cells from pure culture in TSB plus 0.6% YE grown for 11h at 37ºC and 120 rpm (undamaged cells; *X* 25,000), (b) apple juice (undamaged cells; *X* 25,000) and (c) apple juice with 0.8% malic acid (cells with alteration of cytoplasmic content; *X* 25,000) incubated for 24h at 37ºC.

4-CONCLUSIONS

Apple, pear and melon juices inhibit the growth of *L. monocytogenes*, *S.* Enteritidis and *E. coli* O157:H7 at 5ºC by at least 5 days. However, at 20 and 35ºC only apple and pear juices showed that same effect over the pathogens studied, being necessary concentrations of 0.2% of malic acid to inhibit the growth of *L. monocytogenes* and *S.* Enteritidis and 0.4% for *E. coli* O157:H7 in melon juice at those temperatures. On the other hand, higher concentrations of malic acid showed to be effective to reduce and inactivate in more than 5-log cycles of those microorganisms in apple, pear and melon juices. That inactivation depended on the kind of juice, storage temperature and time as well as the microorganism type. According to ours results *E. coli* O157:H7 and refrigeration temperatures (5ºC) should be considered as target microorganism and temperature in the fruit juices preservation when organic acids are used.

Transmission Electron Microscopy studies demonstrated that malic acid got into the *L. monocytogenes*, *S.* Enteritidis and *E. coli* O157:H7 cells generating an agglutination of cytoplasmic content without disruption of cell membrane and causing the death of the microorganisms. This result is a contribution to understanding of antimicrobial action of malic acid against pathogenic microorganisms.

MICs and MBCs found in this study may serve as base to future studies where combination of malic acid with other preservation methods could be considered. In addition, studies of shelf-life and sensory evaluation are recommended to evaluate how could affect the different concentrations of malic acid the fruit juices quality.

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Antimicrobial activity of essential oils on Salmonella Enteritidis, Escherichia coli and Listeria innocua in fruit juices

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ABSTRACT

The antimicrobial properties of essential oils and their derivatives are known since years ago. However, the information published about the minimal effective concentration of essential oils against microorganisms in fruit juices is scarce. In this study, both minimal inhibitory (MIC) and minimal bactericidal (MBC) concentrations of six essential oils **(**lemongrass, cinnamon, geraniol, palmarosa or benzaldehyde**)** against *S.* Enteritidis*, E. coli* and *L. innocua* were determined by the agar and broth dilution methods respectively. All the six essential oils inhibited the microbial (*S.* Enteritidis, *E. coli* and *L. innocua*) growth at a concentration from 1 µl/ml (MIC). These studies lead to choose the three most effective essential oils. Lemongrass, cinnamon and geraniol were found to be most effective to inhibit the growth of the microorganisms and thus used for the MBC analysis. In this last point, significant differences ($p < 0.05$) among essential oils, their concentrations and culture media (apple, pear, melon juices or TSB medium) were found after comparing the results on MBCs for each microorganism. A concentration of 2 µl/ml from lemongrass, cinnamon or geraniol was enough for inactivate *S.* Enteritidis, *E. coli* and *L. innocua* in apple and pear juices. However, in melon juice and TSB medium, concentrations of 8 and 10 µl/ml from cinnamon respectively, or 6 µl/ml from geraniol were necessary to eliminate the three microorganisms, while lemongrass only require 5 µl/ml for inactivate them. These results suggest that essential oils represent a good alternative to eliminate microorganisms which can be a hazard for the consumer in unpasteurized fruit juices. The present study contributes to the knowledge of MBCs of essential oils against pathogenic bacteria on fruit juices.

Key words: Essential oils, fruit juices, *Listeria, Salmonella, E. coli*

INTRODUCTION

Unpasteurized fruit juices have desirable flavor characteristics but short shelf life due to microbial and enzymatic spoilage (*27*). In addition, those foods may be vehicles of pathogenic microorganisms (*2*). Several outbreaks of foodborne disease caused by pathogenic bacteria have been associated with juices (*2, 7, 15, 19*). The widespread production and consumption of juices without thermal process, suggest the need to develop new strategies to protect juices and consumers against pathogens. In many cases, the infective dose is very low and thus, the only presence of the microorganism is enough to cause illness. Traditional thermal process is used to inactivate enzymes and destroy pathogenic microorganisms in juices; however, it can produce important sensorial changes on them.

Antimicrobial agents have been used to inhibit foodborne bacteria and extend the shelf life of processed food (*4, 18*). Herbs and spices, widely used in the food industry as flavors and fragrances, have show additional antimicrobial functions against foodborne pathogens (*16, 21*). Moreover, essential oils (EOs) or their constituents have also demonstrated antimicrobial activity (*3*).

The majority of the EOs are classified as Generally Recognized As Safe (GRAS) according to Food Additive Status List, (*11*). However, their use in foods as preservatives is often limited due to flavor considerations, since effective antimicrobial doses might exceed organoleptically acceptable levels. Therefore, there is an increasing interest for an accurate knowledge of the minimal inhibitory concentrations (MIC) of essential oils to enable a balance between the sensory acceptability and the antimicrobial efficacy (*17*).

Several previous studies have shown that essential oils from cinnamon, geraniol, lemongrass, clove and palmarosa have antimicrobial effect in culture media (*1, 3, 6, 13, 14, 16, 20, 22- 24*). However, there is little information available about MIC or MBC of these oils in fruit juices (*5,12*).

The first objective of this study was to investigate the effectiveness of selected essential oils on survival and growth of microorganisms, which can be used as indicators of the incidence of pathogens in food such as *Salmonella* Enteritidis, *Escherichia coli* and *Listeria innocua* in culture media (tryptone soy agar and tryptone soy broth) and fruit juices with different characteristics (apple, pear and melon). The second objective of this work was to find MIC and MBC of the essential oils that resulted more effective against each microorganism and thus contribute to the knowledge

of the needed concentrations of specific essential oils for preventing the incidence or eliminate pathogenic microorganisms present in fruit juices.

MATERIALS AND METHODS

Microorganisms. Strains of *Salmonella* Enteritidis 1.82 (National Collection of Type Culture (NCTC) 9001, PHLS Central Public Health Laboratory; London, UK), *Escherichia coli* 1.107 (Laboratoire de Répression des Fraudes (LRF); Montpellier, France) and *Listeria innocua* (Laboratoire de Répression des Fraudes (LRF); Montpellier, France) were provided for the culture collections of the Department of Food Technology, University of Lleida, Spain.

Preparation of bacterial cultures for inhibitory tests. The strains of *S.* Enteritidis and *E. coli* were maintained in nutrient agar (NA) (Biokar Diagnostics. Beauvais, France) slants at 5ºC, while the strain of *L. innocua* was in tryptone soy agar (TSA) (Biokar Diagnostics. Beauvais, France) slants at 5ºC. Stock cultures of *S.* Enteritidis and *E. coli* were grown in tryptone soy broth (TSB) (Biokar Diagnostics. Beauvais, France) at 37ºC for 11 hours and 120 rpm (cell in early stationary phase), while stock culture of *L. innocua* was grown in TSB + 0,6% of yeast extract (Biokar Diagnostics. Beauvais, France) at 35ºC for 15 hours and 200 rpm (cell in early stationary phase). The maximum level for each microorganism was 4.8×10^9; 4.0×10^9 and 4.9×10^9 colony forming units / milliliter (CFU/ml) for *S.* Enteritidis, *E. coli* and *L. innocua* respectively. Concentrations were then adjusted to 10^2 - 10^3 or 10^6 CFU/ml using saline peptone water (Scharlau Chemie, S.A. Barcelona, Spain) in agar dilution method and broth dilution assay respectively.

Essential oils (EOs). Essential oils of herbaceous portions of palmarosa (*Cymbopogon martini*) plant whole, clove (*Eugenia caryophyllata*) leaf, cinnamon (*Cinnamomum zeylanicum*) leaf and lemongrass (*Cymbopogon citrates*) were obtained from Aceites Esenciales Dicana (Barcelona, Spain); benzaldehyde (98 %) was obtained from Acros Organics (New Jersey, USA) and geraniol (approx. 98%) from Sigma (Steinhein, Germany).

Antibacterial Assay. MIC of each essential oil against *S.* Enteritidis*, E. coli* and *L. innocua* were determined by the agar dilution method reported by Davidson and Parish (9) in TSA with the following modifications: A short heating (5 min at 100ºC) was applied after adding the essential oil to the agar to facilitate the dissolution of the oil in the media. In addition, 5 ml/l of 2, 3, 5 - Triphenyltetrazolium Chlorid (TTC) (Merck. Darmstadt, Germany) 0.05 % was incorporated into the agar after cooling until 50ºC to facilitate colony recount. The final concentrations of essential oils in the agar were 1, 3, 5 y 10 µl/ml. One hundred micro-liters of cultures stock diluted until 10^2 - 10^3 CFU/ml of each microorganism were spread on the plates. A control of each microorganism was made in TSA without essential oil. Plates were incubated at 35ºC for 24 h. The MIC was considered as the lowest concentration to maintain or reduce the inoculum level.

Based on the results of the first part of this work, lemongrass, cinnamon and geraniol were selected as more effective to be assayed during the determination of the minimum bactericidal concentrations. MBCs were determined by the broth dilution method reported by Davidson and Parish (*9*) in TSB and fruit juices (apple, pear and melon) with the following modifications: a final concentration of 2 % (v/v) Tween 80 (Scharlau Chemie S.A. Barcelona, Spain) was incorporated into the broth or the juices before autoclaving to facilitate miscibility of essential oils in each liquid medium. Nevertheless, Lambert *et al.* (17) reported that MIC or MBC trend to be a function of dispersion agent used. Since, the MIC or MBC could be lower when this agent either is not used or is used scarcely.

The fruits were washed, peeled, cut into pieces and blended (Ufesa Model BP 4512. Victoria, Spain). The obtained juices were centrifuged (Avanti™ J-25 Centrifuge Beckman, USA) at 12.500 rpm x 15 min at 4ºC. The supernatant was filtered and autoclaved. Five hundred microliter aliquot of bacterial suspension (*S.* Enteritidis*, E. coli* or *L. innocua*) at 10^6 CFU/ml and each of the essential oils to give a final concentration of 2, 3, 5, 6, 8 or 10 µl/ml were added to each tube containing 450 µl of sterile TSB, apple, pear or melon juices with 2 % of Tween 80. A control of each medium (TSB, apple, pear or melon juices with 2 % of Tween 80) without essential oils was made. These experiments were conducted twice and in duplicate for each medium, essential oil and concentration.

The tubes were incubated at 35ºC for 24 h to simulate abuse conditions by consumers in the handling of these kind of products, and then a 100 µl aliquot was spread on TSA plates to check bacterial survival and growth. Before establishing the MBC, an examination of injured and death

cells was made by adding a 500 µl aliquot of medium from the tubes incubated previously to tubes with 450 µl of fresh medium (TSB) without EOs and incubating at 35ºC for 24 h. The MBC was considered to be the EO concentration where growth in plate or fresh media was not detected.

Determination of pH. Apple, pear, melon juices and TSB medium pH was determined using a Microprocessor pH meter Hanna Instruments PH210 (Vernon Hills, USA).

Confocal Scanning Laser Microscopy (CSLM). The analysis was carried out according to Wierzchos *et al*. (*26*) and De los Rios *et al.* (*10*) methodology. This methodology allows distinguishing between living and dead cells through the application of the Kit L-13152 (Molecular Probes) which have two proprietary nucleic acid stains that differ in their ability to penetrate bacterial cell membranes. The green fluorescence nucleic acid stain, SYTO 9, labels all cells and the red fluorescence nucleic acid stain, propidium iodide, only penetrates cells with damaged membranes and quenches the green SYTO 9 stain. Green cells are alive and red cells are dead. This technique may be used to know of a direct and rapid form the bactericidal effect of any substance against the microorganisms.

Transmission Electron Microscopy (TEM). For transmission electron microscopy, *S*. Enteritidis cells were cultured for 24 h in TSB medium, apple juice and apple juice with lemongrass (5 µl/ml). Afterwards, they were fixed in glutaraldehyde (2.5 % in 0.1 M phosphate buffer, pH 7.4) for 1 h, rinsed three times for 10 min with 0.1 M phosphate buffer (pH 7.4) and post fixed with 1 % osmium tetraoxide for 2 h a 4°C. After fixation, the cells were rinsed three times for 10 min with 0.1 M phosphate buffer (pH 7.4) and then dehydrated using 30, 50, 70, and 95 % acetone sequentially for 15 min each. Next, the cells were dehydrated three times for 30 min with 100 % acetone. After dehydration, the cells were treated with propylene oxide twice for 10 min a 4°C. The cells were sequentially infiltrated with a mixture of propylene oxide: Durcupan's ACM Epoxy Resin (3:1, 1:1, and 1:3) for 45 min. Polymerization of the resin to form specimen blocks was performed in an oven at 60°C for 72 h. The specimen blocks were hand trimmed with a razor blade and sectioned with a diamond knife in a Reichert Ultracut R ultramicrotome (Leica, Wetzler, Germany). Thin sections (70 to 80 nm) were placed on 300-mesh copper grids. The sections were stained for 15-20 min in uranyl: ethyl alcohol (1:1), after washed 3 times for 2 min and then incubated in a drop of Reynold's lead

citrate and examined using an EM 910 Zeiss transmission electron microscope, Germany.

Statistical Analysis. The results of the MBCs were analyzed by Multifactor ANOVA, using STATGRAPHICS Plus, 5.1. MBCs obtained for each microorganism were analyzed independently, and the evaluated factors were type of essential oil (lemongrass, cinnamon and geraniol), essential oil concentration (0, 2, 3, 5, 6, 8 and 10 µl/ml) and type of culture media (apple, pear, melon juices and TSB).

RESULTS AND DISCUSSION

Minimal inhibitory concentration (MIC). The evaluated essential oils demonstrated to have antibacterial activity against the three tested microorganisms but in different degrees. Lemongrass, cinnamon, geraniol, clove and benzaldehyde resulted more effective than palmarosa to inhibit growing of *E. coli*. The growth *of S.* Enteritidis was better inhibited by lemongrass, cinnamon, geraniol and palmarosa than by clove or benzaldehyde while the greatest inhibition of the growth of *L. innocua* was found when using lemongrass, geraniol and palmarosa (Table 1).

The MIC against *E. coli, S*. Enteritidis and *L. innocua* showed by the six studied essential oils resulted to be always the same (1 µl/ml) (Table 1). Similar studies with *S.* Enteritidis and *L. innocua* were not found in the literature, and thus other *Salmonella* and *Listeria* species have been selected as a reference for comparison. A similar behavior to *E. coli* and *S.* Typhimurium (strain VR-19) was reported by Pattnaik *et al.,* (*22*) who found a MIC of 1.66 µl/ml of lemongrass and palmarosa against *E. coli,* whereas for *S.* Typhimurium (VR-19), they encountered a MIC of 1.66 µl/ml and 0.80 µl/ml respectively. However, Hammer *et al*. (*14*) needed a higher concentration of lemongrass (2.5 µl/ml), palmarosa (5 µl/ml) and clove (>20 µl/ml) to inhibit *S*. Typhimurium (ATCC 13311). On the other hand, Kim *et al.* (*16*) found similar MIC of geraniol to ours, reporting concentrations of 0.5 µl/ml and 1 µl/ml of geraniol against *S.* Typhimurium and *L. monocytogenes* respectively. The differences found among results showed by diverse researchers may be consequence of the spices nature, the specific strains or the microorganism resistances.

Table 1. Inhibitory activity of some essential oils, against *Escherichia coli, Salmonella* Enteritidis and *Listeria innocua.*

EO	Concentration (µl/ml)	Microorganism (CFU/ml)[a]		
		Escherichia coli	***Salmonella* Enteritidis**	***Listeria innocua***
Lemongrass	0	6.20×10^2	9.85×10^2	5.90×10^3
	1	4.25×10^2	4.40×10^2	3.88×10^3
	3	< 10	6.5×10^1	< 10
	5	< 10	< 10	< 10
	10	< 10	< 10	< 10
Cinnamomum zeylanicum (Cinnamon)	0	6.20×10^2	9.85×10^2	5.90×10^3
	1	3.55×10^2	5.20×10^2	3.60×10^3
	3	< 10	< 10	3.08×10^3
	5	< 10	< 10	< 10
	10	< 10	< 10	< 10
Geraniol	0	6.20×10^2	9.85×10^2	5.90×10^3
	1	3.60×10^2	1.11×10^3	4.50×10^3
	3	< 10	< 10	< 10
	5	< 10	< 10	< 10
	10	< 10	< 10	< 10
Cymbopogon martini (Palmarosa)	0	6.20×10^2	9.85×10^2	5.90×10^3
	1	5.00×10^2	1.07×10^3	4.48×10^3
	3	2.50×10^2	< 10	< 10
	5	1.00×10^1	< 10	< 10
	10	< 10	< 10	< 10
Eugenia caryophyllata (Clove)	0	6.20×10^2	9.85×10^2	5.90×10^3
	1	4.65×10^2	3.85×10^2	4.06×10^3
	3	< 10	1.00×10^2	2.1×10^2
	5	< 10	< 10	< 10
	10	< 10	< 10	< 10
Benzaldehyde	0	6.20×10^2	9.85×10^2	5.90×10^3
	1	5.95×10^2	4.25×10^2	5.72×10^3
	3	< 10	1.55×10^2	3.68×10^3
	5	< 10	< 10	3.12×10^3
	10	< 10	< 10	< 10

[a] < 10 Not detected

The methodology applied has great influence on the results. Different methods for detecting the MICs and inoculum volumes have been used. In this way, Hammer *et al*. (*14*) used 1-2 µl spots containing the microorganism and applied the agar dilution method, whereas Kim *et al*. (*16*) employed 200 µl aliquot of bacterial suspension and the broth dilution method. The inoculum is sometimes dotted and other times streaked onto the agar surface. On the other hand, different solvents to incorporate the EOs in the medium have been used, thus Pattnaik *et al*. (*22*) used sodium taurocholate, while Hammer *et al*. (*14*) used tween 20 to facilitate miscibility of essential oils within the medium.

In addition to specific strains, specie resistances and methodology applied, the MIC definition used by each research group is a factor that may complicate the comparison among published data. The definition of MIC itself differs among publications. Some researchers consider MIC as the lowest concentration resulting in maintenance or reduction of inoculum viability (*3*), while others define it as the lowest concentration resulting in a significant decrease (> 90 %) (*8*) or complete destruction of the inoculum viability (*25*). The application of different concepts and methodologies may influence significantly the results and bring, as a consequence, the comparison among results more difficult.

Minimal bactericidal concentration (MBC). Lemongrass, cinnamon and geraniol were selected as more effectives against the evaluated microorganisms; based in the results of the MIC made previously**.**

Similarly to the MIC assay, differences among the effectivity of lemongrass, cinnamon or geraniol against the three microorganisms were observed (Tables 2, 3 and 4). Statistical analysis were made independently for each microorganism and showed significant differences ($p< 0.05$) among essential oils, their concentrations and culture media.

Bactericidal activity varied among culture media for the same strain. Higher concentrations of essential oils were required to inactivate populations of each strain in melon juice and TSB than apple and pear media (Tables 2, 3 and 4). These results are supported by the statistical analysis, which show significant differences ($p< 0.05$) between the counts of microorganism obtained from different culture media (TSB, melon, pear and apple juices). Nychas and Skandamis (*21*) reported that the antimicrobial activity of the naturally occurring antimicrobial compounds in plants or their constituents (essential oils for example) is influenced by the culture medium, the temperature of incubation and the inoculum size.

Table 2. Effect of different concentrations of cinnamon, lemongrass and geraniol against *Salmonella* Enteritidis in apple, pear, melon juices or TSB after 24 h of incubation at 35ºC

EO	Concentration (µl/ml)	Media*			
		Apple juice	Pear juice	Melon juice	TSB
Cinnamon	0	$3.01 \pm 0.29^{Ab\alpha}$	$4.13 \pm 0.03^{Ab\alpha}$	$7.88 \pm 0.18^{Ab\beta}$	$8.96 \pm 0.06^{Ab\gamma}$
	2	$< 1^{Aa\alpha}$	$< 1^{Aa\alpha}$	$< 1^{Aa\beta}$	$< 1^{Aa\gamma}$
	3	$< 1^{Aa\alpha}$	$< 1^{Aa\alpha}$	$< 1^{Aa\beta}$	$< 1^{Aa\gamma}$
	5	$< 1^{Aa\alpha}$	$< 1^{Aa\alpha}$	$< 1^{Aa\beta}$	$< 1^{Aa\gamma}$
	6	$< 1^{Aa\alpha}$	$< 1^{Aa\alpha}$	$< 1^{Aa\beta}$	$< 1^{Aa\gamma}$
	8	$< 1^{Aa\alpha}$	$< 1^{Aa\alpha}$	$< 1^{Aa\beta}$	$< 1^{Aa\gamma}$
	10	$< 1^{Aa\alpha}$	$< 1^{Aa\alpha}$	$< 1^{Aa\beta}$	$< 1^{Aa\gamma}$
Lemongrass	0	$3.01 \pm 0.29^{Ab\alpha}$	$4.13 \pm 0.03^{Ab\alpha}$	$7.88 \pm 0.18^{Bb\beta}$	$8.96 \pm 0.06^{Bb\gamma}$
	2	$< 1^{Aa\alpha}$	$< 1^{Aa\alpha}$	$2.78 \pm 0.01^{Bc\beta}$	$3.41 \pm 0.02^{Bc\gamma}$
	3	$< 1^{Aa\alpha}$	$< 1^{Aa\alpha}$	$1.50 \pm 0.71^{Bd\beta}$	$3.42 \pm 0.02^{Bc\gamma}$
	5	$< 1^{Aa\alpha}$	$< 1^{Aa\alpha}$	$< 1^{Ba\beta}$	$< 1^{Ba\gamma}$
	6	$< 1^{Aa\alpha}$	$< 1^{Aa\alpha}$	$< 1^{Ba\beta}$	$< 1^{Ba\gamma}$
	8	$< 1^{Aa\alpha}$	$< 1^{Aa\alpha}$	$< 1^{Ba\beta}$	$< 1^{Ba\gamma}$
	10	$< 1^{Aa\alpha}$	$< 1^{Aa\alpha}$	$< 1^{Ba\beta}$	$< 1^{Ba\gamma}$
Geraniol	0	$3.01 \pm 0.29^{Ab\alpha}$	$4.13 \pm 0.03^{Ab\alpha}$	$7.88 \pm 0.18^{Ab\beta}$	$8.96 \pm 0.06^{Ab\gamma}$
	2	$< 1^{Aa\alpha}$	$< 1^{Aa\alpha}$	$< 1^{Aa\beta}$	$< 1^{Aa\gamma}$
	3	$< 1^{Aa\alpha}$	$< 1^{Aa\alpha}$	$< 1^{Aa\beta}$	$< 1^{Aa\gamma}$
	5	$< 1^{Aa\alpha}$	$< 1^{Aa\alpha}$	$< 1^{Aa\beta}$	$< 1^{Aa\gamma}$
	6	$< 1^{Aa\alpha}$	$< 1^{Aa\alpha}$	$< 1^{Aa\beta}$	$< 1^{Aa\gamma}$
	8	$< 1^{Aa\alpha}$	$< 1^{Aa\alpha}$	$< 1^{Aa\beta}$	$< 1^{Aa\gamma}$
	10	$< 1^{Aa\alpha}$	$< 1^{Aa\alpha}$	$< 1^{Aa\beta}$	$< 1^{Aa\gamma}$

*Means ± standard deviation of plate counts from two experiments, each in duplicate (n = 4) expressed as Log_{10} CFU/ml.
Different capital letters (A, B) represent significant differences ($p < 0.05$) among essential oils type by each medium. Different small letters different (a, b, c, d) represent significant differences ($p < 0.05$) among essential oils concentrations by each essential oil and medium. Different Greek letters (α, β, γ) represent significant differences ($p < 0.05$) among culture media by each essential oil.

Table 3. Effect of different concentrations of cinnamon, lemongrass and geraniol against *Escherichia coli* in apple, pear, melon juices or TSB after 24 h of incubation at 35ºC

EO	Concentration (µl/ml)	Media*			
		Apple juice	**Pear juice**	**Melon juice**	**TSB**
Cinnamon	0	$3.39 \pm 1.19^{Ab\alpha}$	$4.36 \pm 0.88^{Ab\alpha}$	$8.70 \pm 0.01^{Ab\beta}$	$9.14 \pm 0.02^{Ab\beta}$
	2	$< 1^{Aa\alpha}$	$< 1^{Aa\alpha}$	$< 1^{Aa\beta}$	$< 1^{Aa\beta}$
	3	$< 1^{Aa\alpha}$	$< 1^{Aa\alpha}$	$< 1^{Aa\beta}$	$< 1^{Aa\beta}$
	5	$< 1^{Aa\alpha}$	$< 1^{Aa\alpha}$	$< 1^{Aa\beta}$	$< 1^{Aa\beta}$
	6	$< 1^{Aa\alpha}$	$< 1^{Aa\alpha}$	$< 1^{Aa\beta}$	$< 1^{Aa\beta}$
	8	$< 1^{Aa\alpha}$	$< 1^{Aa\alpha}$	$< 1^{Aa\beta}$	$< 1^{Aa\beta}$
	10	$< 1^{Aa\alpha}$	$< 1^{Aa\alpha}$	$< 1^{Aa\beta}$	$< 1^{Aa\beta}$
Lemongrass	0	$3.39 \pm 1.19^{Ab\alpha}$	$4.36 \pm 0.88^{Ab\alpha}$	$8.70 \pm 0.01^{Ab\beta}$	$9.14 \pm 0.02^{Ab\beta}$
	2	$< 1^{Aa\alpha}$	$< 1^{Aa\alpha}$	$< 1^{Aa\beta}$	$< 1^{Aa\beta}$
	3	$< 1^{Aa\alpha}$	$< 1^{Aa\alpha}$	$< 1^{Aa\beta}$	$< 1^{Aa\beta}$
	5	$< 1^{Aa\alpha}$	$< 1^{Aa\alpha}$	$< 1^{Aa\beta}$	$< 1^{Aa\beta}$
	6	$< 1^{Aa\alpha}$	$< 1^{Aa\alpha}$	$< 1^{Aa\beta}$	$< 1^{Aa\beta}$
	8	$< 1^{Aa\alpha}$	$< 1^{Aa\alpha}$	$< 1^{Aa\beta}$	$< 1^{Aa\beta}$
	10	$< 1^{Aa\alpha}$	$< 1^{Aa\alpha}$	$< 1^{Aa\beta}$	$< 1^{Aa\beta}$
Geraniol	0	$3.39 \pm 1.19^{Ab\alpha}$	$4.36 \pm 0.88^{Ab\alpha}$	$8.70 \pm 0.01^{Bb\beta}$	$9.14 \pm 0.02^{Bb\beta}$
	2	$< 1^{Aa\alpha}$	$< 1^{Aa\alpha}$	$3.40 \pm 0.02^{Bc\beta}$	$3.44 \pm 0.01^{Bc\beta}$
	3	$< 1^{Aa\alpha}$	$< 1^{Aa\alpha}$	$3.36 \pm 0.02^{Bc\beta}$	$3.41 \pm 0.02^{Bc\beta}$
	5	$< 1^{Aa\alpha}$	$< 1^{Aa\alpha}$	$2.36 \pm 0.13^{Bd\beta}$	$1.25 \pm 0.07^{Bd\beta}$
	6	$< 1^{Aa\alpha}$	$< 1^{Aa\alpha}$	$< 1^{Ba\beta}$	$< 1^{Ba\beta}$
	8	$< 1^{Aa\alpha}$	$< 1^{Aa\alpha}$	$< 1^{Ba\beta}$	$< 1^{Ba\beta}$
	10	$< 1^{Aa\alpha}$	$< 1^{Aa\alpha}$	$< 1^{Ba\beta}$	$< 1^{Ba\beta}$

*Means ± standard deviation of plate counts from two experiments, each in duplicate (n = 4) expressed as Log_{10} CFU/ml.
Different capital letters (A, B) represent significant differences ($p < 0.05$) among essential oils type by each medium. Different small letters different (a, b, c, d) represent significant differences ($p < 0.05$) among essential oils concentrations by each essential oil and medium. Different greek letters (α, β) represent significant differences ($p < 0.05$) among culture media by each essential oil.

Table 4. Effect of different concentrations of cinnamon, lemongrass and geraniol against *Listeria innocua* in apple, pear, melon juices or TSB after 24 h of incubation at 35ºC

EO	Concentration (µl/ml)	Media*			
		Apple juice	**Pear juice**	**Melon juice**	**TSB**
Cinnamon	0	$1.80 \pm 0.37^{Ab\alpha}$	$2.01 \pm 0.50^{Ab\alpha}$	$8.31 \pm 0.19^{Bb\beta}$	$9.02 \pm 0.03^{Bb\beta}$
	2	$< 1^{Aa\alpha}$	$< 1^{Aa\alpha}$	$3.40 \pm 0.01^{Bc\beta}$	$3.41 \pm 0.01^{Bc\beta}$
	3	$< 1^{Aa\alpha}$	$< 1^{Aa\alpha}$	$3.39 \pm 0.03^{Bc\beta}$	$3.40 \pm 0.02^{Bc\beta}$
	5	$< 1^{Aa\alpha}$	$< 1^{Aa\alpha}$	$3.38 \pm 0.02^{Bc\beta}$	$3.39 \pm 0.02^{Bc\beta}$
	6	$< 1^{Aa\alpha}$	$< 1^{Aa\alpha}$	$3.06 \pm 0.19^{Bc\beta}$	$3.39 \pm 0.01^{Bc\beta}$
	8	$< 1^{Aa\alpha}$	$< 1^{Aa\alpha}$	$< 1^{Ba\beta}$	$1.85 \pm 0.21^{Bd\beta}$
	10	$< 1^{Aa\alpha}$	$< 1^{Aa\alpha}$	$< 1^{Ba\beta}$	$< 1^{Ba\beta}$
Lemongrass	0	$1.80 \pm 0.37^{Ab\alpha}$	$2.01 \pm 0.50^{Ab\alpha}$	$8.31 \pm 0.19^{Ab\beta}$	$9.02 \pm 0.03^{Ab\beta}$
	2	$< 1^{Aa\alpha}$	$< 1^{Aa\alpha}$	$< 1^{Aa\alpha}$	$< 1^{Aa\alpha}$
	3	$< 1^{Aa\alpha}$	$< 1^{Aa\alpha}$	$< 1^{Aa\alpha}$	$< 1^{Aa\alpha}$
	5	$< 1^{Aa\alpha}$	$< 1^{Aa\alpha}$	$< 1^{Aa\alpha}$	$< 1^{Aa\alpha}$
	6	$< 1^{Aa\alpha}$	$< 1^{Aa\alpha}$	$< 1^{Aa\alpha}$	$< 1^{Aa\alpha}$
	8	$< 1^{Aa\alpha}$	$< 1^{Aa\alpha}$	$< 1^{Aa\alpha}$	$< 1^{Aa\alpha}$
	10	$< 1^{Aa\alpha}$	$< 1^{Aa\alpha}$	$< 1^{Aa\alpha}$	$< 1^{Aa\alpha}$
Geraniol	0	$1.80 \pm 0.37^{Ab\alpha}$	$2.01 \pm 0.50^{Ab\alpha}$	$8.31 \pm 0.19^{Cb\beta}$	$9.02 \pm 0.03^{Cb\beta}$
	2	$< 1^{Aa\alpha}$	$< 1^{Aa\alpha}$	$3.40 \pm 0.00^{Cc\beta}$	$3.36 \pm 0.01^{Cc\beta}$
	3	$< 1^{Aa\alpha}$	$< 1^{Aa\alpha}$	$3.34 \pm 0.05^{Cc\beta}$	$3.37 \pm 0.02^{Cc\beta}$
	5	$< 1^{Aa\alpha}$	$< 1^{Aa\alpha}$	$1.30 \pm 0.17^{Cd\beta}$	$2.23 \pm 0.39^{Cd\beta}$
	6	$< 1^{Aa\alpha}$	$< 1^{Aa\alpha}$	$< 1^{Ca\beta}$	$< 1^{Ca\beta}$
	8	$< 1^{Aa\alpha}$	$< 1^{Aa\alpha}$	$< 1^{a\beta}$	$< 1^{Ca\beta}$
	10	$< 1^{Aa\alpha}$	$< 1^{Aa\alpha}$	$< 1^{a\beta}$	$< 1^{Ca\beta}$

*Means ± standard deviation of plate counts from two experiments, each in duplicate (n = 4) expressed as Log_{10} CFU/ml.
Different capital letters (A, B, C) represent significant differences ($p < 0.05$) among essential oils type by each medium. Different small letters different (a, b, c, d) represent significant differences ($p < 0.05$) among essential oils concentrations by each essential oil and medium. Different greek letters (α, β) represent significant differences ($p < 0.05$) among culture media by each essential oil.

Essential oils of cinnamon, lemongrass and geraniol showed to be effective at 2 µl/ml concentration in apple and pear juice for the three microorganisms. Similar results were reported by Ceylan *et al.* (*5*), who evaluated the antimicrobial activity and synergistic effect of cinnamon with sodium benzoate or potassium sorbate in controlling *E. coli* O157:H7 in apple juice and demonstrated that cinnamon exhibited significant antimicrobial activity against *E. coli* O157:H7 in apple juice at 1, 2 and 3 µl/ml concentration, and its antimicrobial activity increased with increasing concentrations of cinnamon in apple juice at both 8ºC and 25ºC.

On the other hand, a bactericidal or, in some case, inhibitory effect on the microorganisms was observed in pear and apple juices without essential oil (control). In contrast, melon juice or TSB media did not show any effect. Differences among culture media could be consequence of the pH (apple 4.20 ± 0.02, pear 3.97 ± 0.01, melon 5.91 ± 0.02 and TSB 7.51 ± 0.01), since the influence of pH on bacteria growth is well known. However, other factors as some self constituents of each fruit may play a role as inhibitory or bactericidal agent.

Lemongrass resulted different than cinnamon or geraniol in the Multiple Range Tests for *S.* Enteritidis, showing to be the least effective of those three essential oils in melon and TSB media. A concentration of 2 µl/ml of geraniol or cinnamon was enough for inactivate *S.* Enteritidis population, while 5 µl/ml of lemongrass were required to obtain the same effect in both media (Table 2). Kim *et al.* (*16*) reported a lower concentration of geraniol (0.5 µl/ml) for *S.* Typhimurium. It is important to highlight that they worked with different specie and this is a factor that may influence the results.

A concentration of 6 µl/ml of geraniol was needed to destroy *E. coli* population inoculated in melon juice or TSB as growth media, whereas 2 µl/ml of lemongrass or cinnamon were sufficient for eliminate the microorganism (Table 3). Kim *et al.* (*16*) reported a lowest MBC of geraniol (0.5µl/ml) for *E. coli* and *E. coli* O157:H7. Those researchers used a turbidimetric analysis to determine MBC values. The method is not sensitive enough because the detection limit is high. A minimal concentration of $10^6 - 10^7$ CFU/ml of a bacterial culture is required for being detected by the spectrophotometer. Thus, a bacterial culture with a concentration below 10^5 CFU/ml, and actively growing, is undetected. In contrast to turbidimetric analysis, the plate count method, which was used in this work, has a level of 10^2 CFU/ml as detection limit.

On the other hand, Lemongrass resulted to be useful against *L. innocua* in juice melon or TSB media in a concentration of 2 µl/ml,

whereas geraniol was effective at 6 µl/ml and cinnamon was successful at 8 µl/ml in melon juice, and 10 µl/ml in TSB medium (Table 4). Lower concentration of geraniol (1 µl/ml) was indicated by Kim *et al.* (*16*) for kill *L. monocytogenes*.

Friedman *et al.* (*13*) found that strains of *E. coli*, *S. enterica* and *L. monocytogenes* exhibited similar susceptibilities to inactivation by essential oils or oil compounds. However, ours results demonstrated differences among the behavior of *S.* Enteritidis, *E. coli* and *L. innocua* in the presence of cinnamon, geraniol or lemongrass oil, showing different MBC. These results show that the specie or strain has a significant influence on the effect of different antimicrobial substances.

In general, lemongrass demonstrated to be more effective than cinnamon and geraniol in melon juice and TSB media for the three studied microorganisms, since a concentration of 5 µl/ml eliminate all of them, whereas higher concentrations of cinnamon (10 µl/ml) or geraniol (6 µl/ml) were required to obtain a similar effect.

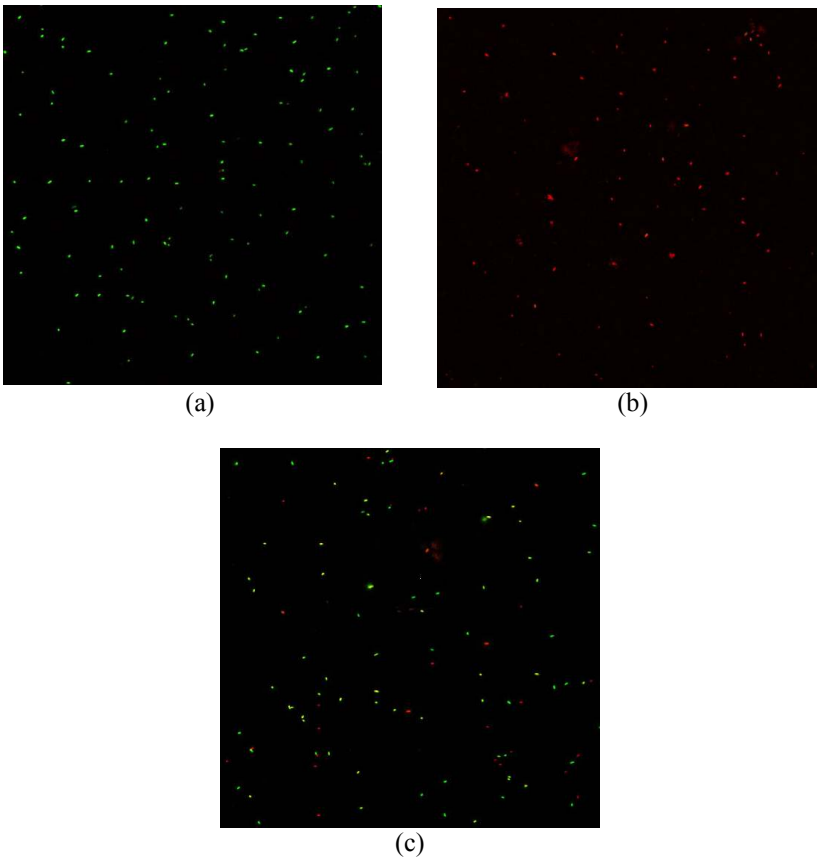
The biggest differences in the bactericidal effects of essential oils within culture medium were observed between the media with and without essential oil (Tables 2, 3 and 4). A concentration of 2 µl/ml of lemongrass, cinnamon or geraniol showed to have a strong effect against the three studied microorganisms in apple and pear media and hence, an increase of oil concentration did not lead to a higher effectiveness.

In order to see the effects of the exposure to essential oils on *S.* Enteritidis, *E. coli* and *L. innocua,* cells of each microorganism cultured for 24 h in TSB media, apple, pear and melon juices were prepared to be observed by Confocal Scanning Laser Microscopy (CSLM) according to Wierzchos *et al.* (*26*) and De los Rios *et al.* (*10*) methodology, and Transmission Electron Microscopy (TEM).

Figure 1 shows the effects of lemongrass (5 µl/ml) added to apple juice on the *S.* Enteritis cells using CSLM. The Kit L-13152 (Molecular probes) Live/Dead ® BacLight™ permitted to distinguish between dead or damaged cells in red color and the alive in green color. This figure illustrates in a rapid and direct form, the bactericidal effect of the EOs on microorganisms. A smaller percentage of fluorescent red cells was observed in the fruit juice sample without essential oil (Fig. 1c), whereas the addition of 5 µl/ml lemongrass to the fruit juice sample resulted in 100 % fluorescent red cells (Fig. 1b), indicating that the EO deteriorated the cellular membrane and converted intracellular nucleic acids approachable to propidium iodide for stained of red. These results are in agreement with those of Lambert *et al.* (17), who observed the same behavior in TSB

medium to evaluate the MIC and mode action of oregano essential oil, thymol and carvacrol.

On the other hand, figure 2 provides images in detail of the essential oil effects on cellular structure using TEM.



(a) (b)

(c)

Figure 1.- Confocal Scanning Laser Microscopy image of *S.* Enteritidis cells from (a) Live cells (green) of a pure culture in TSB grew by 11 h a 37ºC and 120 rpm, (b) Dead cells (red) in apple juice with lemongrass (5 µl/ml) and (c) Live/dead cells in apple juice without addition of EO, incubated by 24 h to 37ºC. Using kit of viability bacterial L-13152 (Molecular probes) LIVE/DEAD® BacLight™ and wavelength of 515-545 nm, 570 nm and 488 nm respectively.

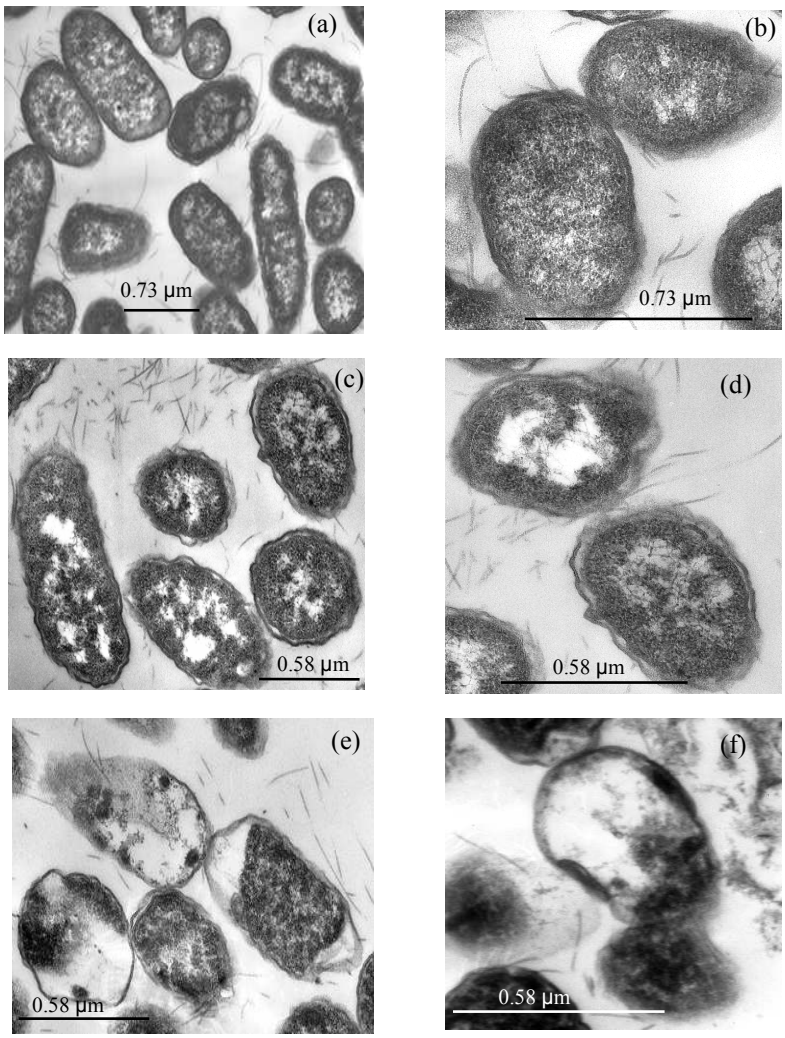


Figure 2.- Transmission Electron Microscopy micrographs of *S.* Enteritidis cells from pure culture in TSB (*a* and *b*) grew by 11 h a 37ºC and 120 rpm (undamaged cells, *X20,000*), Apple juice without addition of EO (*c* and *d*) (cells with alteration of cytoplasmic content and intact cells, *X25,000*), and Apple juice with lemongrass (5 µl/ml) (e and f) incubated by 24 h to 37ºC (cells with structural damages and leakage of cellular content, *X25,000*).

Changes were observed in the cytoplasm of *S*. Enteritidis cells inoculated in apple juice without lemongrass, which show clear zones caused by the action of the pH or some constituent of juice, while damages in the membrane cellular, including disruption of the same and leakage of cell content were found in cells cultivated in apple juice with lemongrass. Results may be explained by the mechanisms of EOs action proposed by different researchers, who consider the hydrophobicity of EOs and their components to be an important characteristic, which enables them to spread through the lipids of the bacterial cell membrane and mitochondria, disturbing the structures and rendering them more permeable causing leakage of ions and other cell contents, thus bringing about an extensive loss of cell contents or the exit of critical molecules and ions, leading to death (*3*).

CONCLUSIONS

EOs of lemongrass, geraniol, cinnamon, clove, palmarosa and benzaldehyde inhibited effectively the *S*. Enteritidis, *E. coli* and *L. innocua* growth at a concentration of at least 1 µl/ml. These previous studies were useful to choose the essential oils more effective against the evaluated microorganisms. Hence, lemongrass, cinnamon and geraniol were chosen for MBC analysis. A concentration of 2 µl/ml from these essential oils was enough for inactivate *S*. Enteritidis, *E. coli* and *L. innocua* in apple and pear juices. On the other hand, lemongrass oil showed to be more effective than cinnamon and geraniol in melon juice and TSB media for the studied microorganisms, since a concentration of 5 µl/ml was enough to eliminate the three microorganisms, whereas higher concentrations of cinnamon (8 and 10 µl/ml) or geraniol (6 µl/ml) were required to obtain the same effect.

This work offers a contribution to the knowledge of MBC of lemongrass, cinnamon and geraniol necessary to avoid the incidence or eliminate pathogenic bacterial as *Salmonella*, *Listeria* and *E. coli* present in unpasteurized fruit juices, whose contamination is originated direct or indirectly via of animals or insects, soil, water, dirty equipment, and human handling.

Results suggest that essential oils represent a good alternative to eliminate microorganisms which can be a hazard for the consumer of unpasteurized fruit juices and be useful to prevent the risk of foodborne illness caused by these kind of products.

Further studies should be conducted to evaluate the sensory aspects of using these natural compounds.

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Use of malic acid and quality stabilizing compounds to assure the safety of fresh-cut "Fuji" apples by inactivation of Listeria monocytogenes, Salmonella Enteritidis and Escherichia coli O157:H7

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ABSTRACT

The effectiveness of malic acid in combination with quality stabilizing to inactivate *Listeria monocytogenes, Salmonella* Enteritidis and *E. coli* O157:H7 inoculated in fresh-cut "Fuji" apples packaged in air and stored at 5ºC was evaluated. Apple pieces were immersed for 1 min. in solutions containing 1% w/v N-acetyl-L-cysteine, 1% w/v glutathione and 1% w/v calcium lactate with and without 2.5% w/v D-L malic acid to control apple browning, softening and reduce the populations of pathogenic microorganisms. Fresh-cut apples dipped in each solution were then inoculated with *L. monocytogenes, S.* Enteritidis or *E. coli* O157:H7. The use of malic acid in combination with quality stabilizing caused more than 5 $\log_{10}$ CFU/g of *L. monocytogenes, S.* Enteritidis and *E. coli* O157:H7 counts. The results obtained in this work pointed out the potential use of malic acid in combination with quality stabilizing compounds as a good alternative for safety assurance of fresh-cut apples.

Practical applications. The use of organic acids as malic acid as well as quality stabilizing substances in fresh-cut fruits such as N-acetyl-L-cysteine, glutathione and calcium lactate can result suitable to fresh-cut products industry, since they can assure the safety and quality of those products. The main reason for their suitability is their natural origin, thus resulting attractive and healthy for the consumers, who demanding products fresh-like.

Keywords: Apple; *L. monocytogenes*; *S.* Enteritidis; *E. coli* O157:H7; malic acid; N-acetyl-L-cysteine; glutathione; calcium lactate

INTRODUCTION

The consumption of fresh-cut fruits has significantly increased in the recent years as a consequence of consumers' demand towards fresh-like, high-quality and healthy products (Hoover, 1997). However, these products, which omit any effective microbial elimination step, result in foods that may carry some naturally occurring microorganisms, including pathogenic bacteria (USFDA, 2001). On the other hand, Balla and Farkas (2006) mentioned that the shelf-life of fresh-cut fruits is limited by deteriorative processes such as browning, softening and microbial decay. Thus, a minimal processing of fresh products to reduce or destroy spoilage and pathogenic microorganisms without significantly affecting their physicochemical and nutritious attributes is required.

In spite of the low pH of many fruits, including apples, emerging pathogenic bacteria such as *Listeria monocytogenes*, *Salmonella* serovars and some *E. coli* strains could be present and cause public health problems. The incidence and/or survival/growth of *L. monocytogenes*, *Salmonella* serovars and *E. coli* O157:H7 in fresh-cut apples as well in apple juices has been demonstrated (Sado *et al*, 1998; Liao and Sapers, 2000; Harris *et al.*, 2003; Lanciotti *et al.*, 2003; Raybaudi-Massilia *et al*, 2006). Outbreaks of *Salmonella* serovars and *E. coli* O157:H7 as well as recalled by *Listeria monocytogenes* linked with apple derivatives have been reported (Besser *et al.,* 1993; CDC, 1996; Powell and Luedtke, 2000; D'Aoust *et al*, 2001; USFDA, 2001; Harris *et al*., 2003; Eswaranandam *et al.*, 2004; Fan *et al.*, 2005).

Currently, stabilizing treatments are being used by processors to avoid quality losses in fresh-cut products. Studies in this area have demonstrated that N-acetyl-L-cysteine and glutathione are good antibrowning agents (Molnar-Perl and Friedman, 1990; Oms-Oliu *et al.*, 2006; Rojas-Graü *et al.*, 2006; Raybaudi-Massilia *et al*., 2007), while others have indicated that calcium salts could maintain the fruit firmness (Johnston *et al.*, 2002; Alandes *et al*., 2006; Quiles *et al*., 2007) and organic acids can delay microbial growth (Derrickson-Tharrington *et al*., 2005; Eswaranandam *et al*., 2004). All these substances are normally applied as dipping solutions just before packaging; nevertheless, their effects on different pathogenic microorganisms are not well known.

Organic acids have been shown to have important antimicrobial properties. Their antimicrobial activity could be attributed to the reduction of the medium pH, decrease of the intracellular pH by ionization of undissociated acid molecules, chelation of metal ions, disruption of

substrate transport by altering cell membrane permeability and/or reduction of proton motive force (Doores, 1993; Stratford and Eklund, 2003; Eswaranandam *et al*., 2004). Though ascorbic and citric acids have been commonly used as antibrowning agents in fresh-cut fruits as dipping treatments (Lanciotti *et al*. 2003; Soliva-Fortuny and Martín-Belloso, 2003; Soliva-Fortuny *et al*., 2004; Rojas-Graü *et al*., 2007), studies on the effects of malic acid with quality stabilizing substances against pathogenic microorganisms in fresh-cut fruits have not been found in the literature. Malic acid is the main organic acid in apples and its concentration varies widely among apple varieties and cultivars (Eisele and Drake 2005), affecting directly the flavor and possibly the fruit shelf-life. In addition, this acid is a relatively new and emerging acidulant preservative, with the potential to impart excellent sensory property to the fruit products (Raju and Bawa, 2006), which is generally recognized as safe (GRAS) by the FDA (USFDA, 2006).

A combination of N-acetyl-L-cysteine, glutathione, calcium lactate and D-L malic acid have been shown to effectively extend the shelf-life of fresh-cut apples by inhibition of spoilage flora (Raybaudi-Massilia *et al.*, 2007); however, their effects on pathogenic microorganisms have not still been studied. Therefore, safety assurance of fresh-cut fruits while maintaining food quality still needs to be studied.

The aim of this research was to evaluate the effectiveness of a combination of D-L malic acid and N-acetyl-L-cysteine, glutathione, calcium lactate to inactivate *Listeria monocytogenes*, *Salmonella* Enteritidis and *E. coli* O157:H7 inoculated in fresh-cut apples.

MATERIALS AND METHODS

Strain and preparation of bacterial cultures.

Listeria monocytogenes 1.131 (CECT 932) and *Escherichia coli* O157:H7 (CECT 4267) from the Spanish Type Culture Collection of the University of Valencia, Valencia, Spain and *Salmonella* Enteritidis 1.82 (NCTC 9001) from the National Collection of Type Culture of the Central Public Health Laboratory; London, UK, were maintained in tryptone soy agar (TSA) (Biokar Diagnostics. Beauvais, France) slants at 5ºC until its use. Stock cultures of *L. monocytogenes* and *E. coli* O157:H7 were grown in tryptone soy broth (TSB) (Biokar Diagnostics) with 0.6% yeast extract (Biokar Diagnostics); whereas, *S.* Enteritidis was cultured in TSB. *E. coli* O157:H7 and *S.* Enteritidis were incubated at 37ºC with continuous

agitation for 11 hours at 120 rpm, while *L. monocytogenes* was incubated at 35ºC with continuous shaking for 15 hours at 200 rpm to obtain cells in early stationary growth phase. The maximum growth for *L. monocytogenes*, *S.* Enteritidis and *E. coli* O157:H7 was 10^9, colony forming units/milliliter (CFU/ml). Concentrations were then adjusted to 10^8 CFU/ml using saline peptone water (0.1% peptone plus 0.85% NaCl, Scharlau Chemie, S.A. Barcelona, Spain).

Preparation of dipping solutions

Two aqueous solutions containing quality stabilizing compounds with or without malic acid were prepared as follows: 1% (w/v) N-acetyl-L-cysteine (Acros Organics, New Jersey, USA), 1% (w/v) glutathione (Acros Organics), 1% (w/v) calcium lactate pent-hydrate (Scharlau Chemie S. A.) into sterile distilled water (CGLW), and 1% (w/v) N-acetyl-L-cysteine, 1% (w/v) glutathione, 1% (w/v) calcium lactate pent-hydrate and 2.5% (w/v) D-L -malic acid (Scharlau Chemie S. A) into sterile distilled water (CGLW+MA). In addition, sterile distilled water (W) was used as a reference treatment for dipping solutions. N-acetyl-L-cysteine, glutathione, calcium lactate and D-L -malic acid concentrations and time of dipping (1 min) were selected according to previous studies Raybaudi-Massilia *et al.* (2007).

Sample preparation and inoculation.

"Fuji" apples **(***Malus domestica* Borkh) with a pH of 4.26 ± 0.18 (Crison 2001 pH-meter; Crison Instruments S. A., Barcelona, Spain), soluble solids content of 12.3 ± 0.4 % (Atago RX-1000 refractometer; Atago Company Ltd, Japan), total acidity of 0.38 ± 0.09 g malic acid/100ml (B.O.E., 1998) and firmness 12.7 ± 0.9 (TA-TX2 Texture Analyzer, Stable Micro Systems LTD. Surrey, England) provided by ACTEL, Lleida, Spain, were previously sanitized by immersion in a 300 µl/L chlorine solution (pH 6.8) for 5 min, then washed with potable water and finally dried with absorbent paper. Firstly, the poles of the cleaned fruits were cut with a knife, and secondly cylinders of 1.40 cm diameter x 2.00 cm high,taken out from the fruit pulp (approximately 6 cylinders per apple) were obtained with a cylindrical stainless steel hollow instrument with sharpened brink. Apple cylinders were immersed for 1 min into the dipping solutions (CGLW, CGLW + MA and W) with a ratio fruit: solution = 1: 2 and constant agitation using a magnetic stirrer. The excess of liquid was drained for 2 min, then 50 g apple cylinders were put on polypropylene trays of 173 x 129 x 35 mm (Ilpra Systems España, S.L, Barcelona, Spain)

and inoculated by uniformly spreading 500 µl of *L. monocytogenes*, *S* Enteritidis or *E. coli* O157:H7 stock cultures (10^8 CFU/ml) over its entire upper surface with a sterile micropipette to achieve a final concentration of 10^6 CFU/g. The trays were then wrap-sealed with a 64 µm thick polypropylene film with a water vapor permeability of 142.86 fmol $s^{-1}m^{-2}$ kPa^{-1} at 38ºC and 90% R.H., O_2 permeability of 52.38 fmol $s^{-1}m^{-2}$ kPa^{-1} at 23ºC and 0% R.H., and CO_2 permeability of 2.38 fmol s^{-1} m^{-2} kPa^{-1} at 23ºC and 0% R.H. (Tecnopack SRL, Mortara, Italy) using a vacuum compensated packaging machine (ILPRA Food Pack Basic V/6, Ilpra S. CP. Vigevono, Italy). Trays were filled with air, heat sealed, and stored in refrigeration at 5ºC for 30 days. The experiments were carried out twice. A total of 12 trays of fresh-cut apples by dipping condition and microorganism were prepared to be analyzed at 0, 3, 7, 14, 21 and 30 days of refrigerated storage.

Microbiological and chemical analysis.

Microbiological analysis of randomly withdrawn samples of fresh-cut apples treated with the different solutions and inoculated with *L. monocytogenes, S.* Enteritidis and *E. coli* O157:H7 were performed in duplicate at 0, 3, 7, 14, 21 and 30 days of refrigerated storage to assess the effect of malic acid and stabilizing substances on these microorganisms.

The pH of fresh-cut apples was also analyzed (Crison 2001 pH-meter) throughout the 30 days of storage in order to detect differences among treatments and possible changes through the storage time.

***L. monocytogenes*, *S.* Enteritidis and *E. coli* O157:H7 survival.** Fifty grams of inoculated fresh-cut apples were diluted with 450 ml of buffered peptone water (pH 7.2) (Biokar Diagnostics) and homogenized in a masticator (IUL Instruments, Barcelona, Spain) for 1 min. Serial dilutions in saline peptone water were prepared and spread at reason of 0.1 ml on Palcam, Hektoen and MacConkey-Sorbitol agar plates in duplicate for *L. monocytogenes* (ISO 11290-2:1998), *S.* Enteritidis and *E. coli* O157:H7 counts, respectively. The plates were incubated for 24-48 h at 35-37ºC and counts were expressed as Log_{10} CFU/g of sample.

***L. monocytogenes*, *S.* Enteritidis and *E. coli* O157:H7 recovery.** Injured cells of *L. monocytogenes*, *S.* Enteritidis and *E. coli* O157:H7 from fresh-cut apples treated with a dipping solution containing malic acid and quality stabilizing substances were recovered by means of an enrichment process with buffered peptone water (pH 7.2) during 20 min at 35-37ºC.

Subsequently, an aliquot of 0.1 ml of each enrichment medium was spread plated on Palcam, Hektoen and MacConkey-Sorbitol agars for *L. monocytogenes*, *S.* Enteritidis and *E. coli* O157:H7 counts, respectively. Those plates were then incubated for 24-48h at 35-37ºC. The recovery medium was selected according to Liao and Fett (2005), whereas recovery time (20 min) was selected taking into account the generation time of each microorganism from growth curves previously obtained in the laboratory (data not shown); where repairing of injured cell without cellular multiplication was assumed.

Statistical analysis.

Microbiological data expressed as the mean of two duplicated determinations (n= 4) were analyzed to determine whether there were significant differences ($p< 0.05$) among dipping conditions and storage time applying an ANOVA with Multiple Range Test. Those analyses were performed with Statgraphics Centurion XV version 15.1.02 statistical package.

RESULTS AND DISCUSSION

Effect of dipping treatments on *L. monocytogenes* inoculated in fresh-cut apples

L. monocytogenes counts were significantly ($p<0.05$) affected by dipping conditions. Inoculated samples without any added compound (W) did not show significant changes in the bacterial load after processing. Nevertheless, the immersion of the apple pieces in CGLW or CGLW + MA solutions, previously to inoculation, caused significant decreases in the population of *L. monocytogenes* (Fig. 1). A reduction of 0.47 $\log_{10}$ CFU/g was observed in fresh-cut apples immersed in CGLW, whereas more than 5 $\log_{10}$ CFU/g reductions were found in apples treated with a CGLW + MA solution at the samples preparation day (t = 0 days). Studies of survival/growth of *L. monocytogenes* in fresh-cut apples without any added preservative have not been found in the literature; therefore, a comparison with our results is not possible. However, previous studies have demonstrated that dipping of fresh-cut apples in 0.2% citric acid and 1% ascorbic acid has only an inhibitory effect on *L. monocytogenes*, whereas a combination of those substances with volatile molecules such as hexanal, (E)-2-hexanal, and hexyl acetate is highly effective against this

microorganism in fresh-cut apples, since it was not detected for at least 4 days of storage at 20ºC (Lanciotti *et al.*, 2003).

On the other hand, negligible changes in *L. monocytogenes* counts on fresh-cut apples dipped in W were observed during 30 days of storage (Fig. 1), thus demonstrating its psychrotrophic condition (Lou and Yousef, 1999). These results differ from those reported by Corbo *et al.* (2005) who found growth of *L. monocytogenes* inoculated on fresh-sliced "Cactus" pear fruit packed under air or passive-modified atmosphere (65% N_2, 30% CO_2, 5% O_2) during the storage at 4, 8, 12 and 20ºC. In contrast, an additional reduction of 2 $\log_{10}$ CFU/g in fresh-cut apples immersed in CGLW was detected after 7 days of storage, and from that day microbial counts were kept almost constant along the rest of the storage time, whereas *L. monocytogenes* counts on fresh cut apples immersed in CGLW + MA were not detected through storage (Fig. 1).

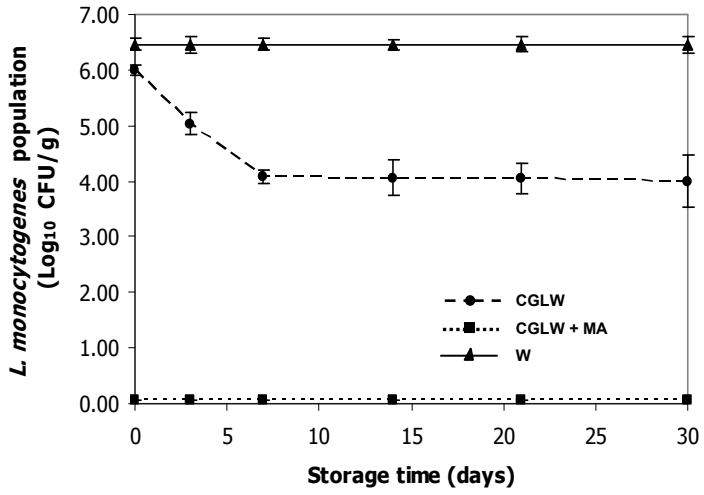

Figure 1.- Reduction of *L. monocytogenes* population in fresh-cut "Fuji" apples dipped in an aqueous solution of N-acetyl-L-cysteine at 1% (w/v), glutathione at 1% (w/v) and calcium lactate at 1% (w/v) **(CGLW),** N-acetyl-L-cysteine at 1% (w/v), glutathione at 1% (w/v), calcium lactate at 1% (w/v) and DL-malic acid 2.5% (w/v) **(CGLW + MA)**, and distilled water **(W)** stored at 5ºC during 30 days. Reported values are the mean of two determination in duplicated (n = 4) ± standard deviation. *L. monocytogenes* counts in fresh-cut apples dipped in CGLW + MA resulted below detection limit (< 2 $\log_{10}$ CFU/g) and undetectable after a recovering step in buffer peptone water at 35-37ºc for 20 minutes.

Effect of dipping treatments on *S.* Enteritidis inoculated in fresh-cut apples

Dipping treatments affected significantly ($p<0.05$) the *S.* Enteritidis behavior in fresh-cut apples (Fig. 2). Microbial counts on fresh-cut apples immersed in W did not have any significant change immediately after processing (t = 0 days). This result is in accordance to that obtained by Liao and Sapers (2000), who did not find changes in *S.* Chester population inoculated on non-treated "Golden delicious" apple disks stored at 8ºC. Likewise, DiPersio *et al.*, (2003) reported that populations of *Salmonella* inoculated on apple slices were not significantly ($p> 0.05$) reduced by immersion in W. In contrast, reductions of up to 1.5 and more than 5 log CFU/g of *S.* Enteritidis were observed in fresh-cut apples dipped in CGLW and CGLW + MA (Fig. 2). A lower reduction (1 to 2 logs) of *S.* Chester

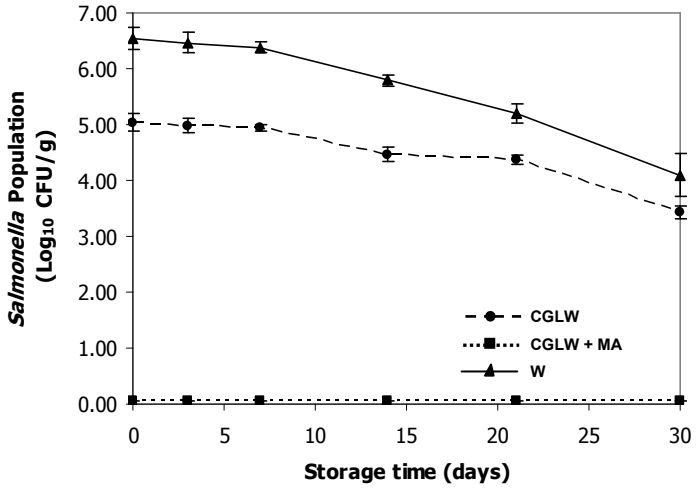

Figure 2.- Reduction of *S.* Enteritidis population in fresh-cut "Fuji" apples dipped in an aqueous solution of N-acetyl-L-cysteine at 1% (w/v), glutathione at 1% (w/v) and calcium lactate at 1% (w/v) **(CGLW)**, N-acetyl-L-cysteine at 1% (w/v), glutathione at 1% (w/v), calcium lactate at 1% (w/v) and DL-malic acid 2.5% (w/v) **(CGLW + MA)**, and distilled water **(W)** stored at 5ºC during 30 days. Reported values are the mean of two determination in duplicated (n = 4) ± standard deviation. *S.* Enteritidis counts in fresh-cut apples dipped in CGLW + MA resulted below detection limit (< 2 $\log_{10}$ CFU/g) and undetectable after a recovering step in buffer peptone water at 35-37ºc for 20 minutes.

in apple disks treated with 6% hydrogen peroxide, 2% trisodium phosphate, 0.36% calcium hypochlorite, and 1.76% sodium hypochlorite was reported by Liao and Sapers (2000). Likewise, Di Persio *et al.*, (2003) reduced *Salmonella* population from 0.9 to 1.1 log CFU/g cycles on apple slices immersed in a citric acid solution at 0.21% for 10 min. The kind of antimicrobial used and concentrations applied could explain those differences observed among those reported results and those obtained in this study, showing malic acid a higher effectiveness than those other compounds reported since higher reductions of *S.* Enteritidis were reached in our case.

On the other hand, significant differences ($p<0.05$) in *S.* Enteritidis counts were found throughout storage time. Survival fractions of 3.44 and 4.09 Log_{10} CFU/g of *S.* Enteritidis in fresh cut apples immersed in CGLW and W, respectively, were observed after 30 days of storage, whereas it was undetectable in fresh-cut apples dipped in CGLW + MA throughout storage time. The reduction in *S.* Enteritidis counts observed throughout storage in fresh-cut apples dipped in W suggests that other factors such as storage temperature (5ºC), competition of the native microflora or headspace gas composition of the trays might influence the pathogen survival. In this way, Raybaudi-Massilia *et al.* (2007) reported significant changes ($p < 0.05$) in headspace gas composition of fresh-cut apples dipped in W without any substance added, indicating that an important decrease of O_2 and a significant increase of CO_2 was observed in fresh-cut apples during 30 days of storage at 5ºC. However, it is well known that *Salmonella* species are facultative anaerobic microorganisms, and thus, headspace gas composition could not be the main cause of the reduction of the *S.* Enteritidis population. Likewise, these authors point out that a meaningful growth of mesophilic, psychrophilic and yeasts and molds populations occurred in fresh-cut apples dipped in W during storage. Therefore, we consider that storage temperature and competition of the native microflora might be the main factors that caused the reduction on *Salmonella* counts through storage when any compound was applied to the fresh-cut apples (W). Similarly, Liao and Sapers, (2000) reported a slight reduction of *S.* Chester on non-treated apple disks stored at 8ºC after 3 days of storage, whereas a significant growth of that microorganism was observed after 3 days of storage at 20ºC.

Effect of dipping treatments on *E. coli* O157:H7 inoculated in fresh-cut apples

Significant reductions of *E. coli* O157:H7 populations were detected in fresh-cut apples treated with CGLW (0.79 Log_{10} CFU/g) or CGLW + MA (2.72 Log_{10} CFU/g) just after preparation (t = 0 days) in comparison with apple pieces dipped in W (Fig. 3). In contrast, Derrickson-Tharrington et al., (2005) reported similar reductions of *E. coli* O157:H7 in apple slices immersed in sterile water or acidic solutions of 2.8% ascorbic acid, 1.7% citric acid or 50% commercial lemon juice with or without preservatives. The results obtained by those authors could be a consequence of the used sequence in the sample preparation, where population of *E. coli* O157:H7 was first inoculated on the surface of the cut apples and then immersed in water or acidic solutions; therefore, microbial reductions may be caused by the washing of the fresh-cut fruits surface during dipping treatments more than by the effect of the added substances. In our case, the apple pieces were firstly treated with dipping solutions and then inoculated with *E. coli* O157:H7, thus ensuring that the obtained microbial reductions were caused by effect of the substances added. In addition, microbial reductions of *E. coli* O157:H7 reported by Derrickson-Tharrington et al., (2005) in apple slices treated with acidic solutions of ascorbic acid, citric acid or commercial lemon juice were lower (0.9 to 1.3 Log CFU/g) than those found in our study with CGLW + MA (2.72 Log_{10} CFU/g), which is probably due to the kind and concentration of the added substances. Eswaranandan et al. (2004) indicated that malic acid was more effective than citric and tartaric acid to reduce pathogenic microorganism populations as a consequence of its lower molecular weight, which may facilitate the entrance of malic acid into microbial cell.

On the other hand, populations of *E. coli* O157:H7 could survive on apple pieces treated with CGLW or W during 30 days of storage, whereas the microorganisms was completely inhibited beyond the third day of storage (Fig. 3) on apples dipped in CGLW + MA. Nonetheless, significant reductions ($p < 0.05$) in *E. coli* O157:H7 populations through the storage time in apple pieces dipped in CGLW or W were observed. These results demonstrate that the storage temperature (5ºC) and/or the native microflora may have a significant effect on *E. coli* O157:H7 inoculated on fresh-cut apples, as occurred with *S.* Enteritidis, since the population of this microorganism on apple pieces treated with water also decreased during storage at 5ºC.

Fisher and Golden, (1998) indicated that *E. coli* O157:H7 was able to survive in non-treated ground Golden Delicious, Red Delicious, Rome and Winesap apples stored at 4ºC during 18 days, observing a slight decrease of microbial populations through the storage time. In contrast, Corbo et al., (2005) reported *E. coli* O157:H7 growth in Cactus-pears fruit during 14 days of storage at 4ºC.

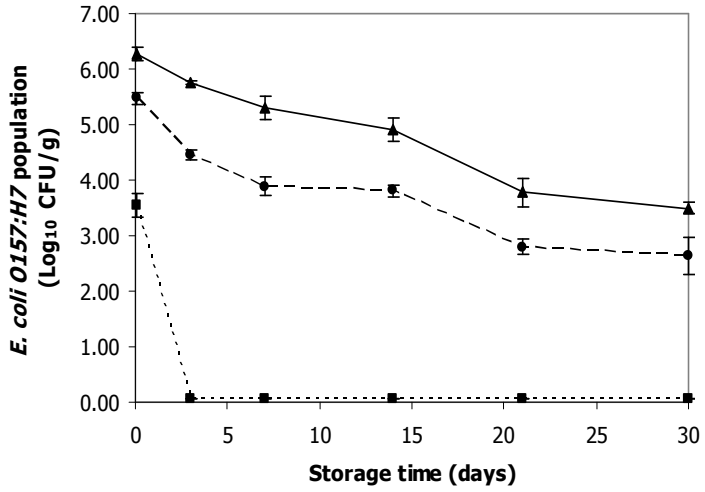

Figure 3.- Reduction of *E. coli* O157:H7 population in fresh-cut "Fuji" apples dipped in an aqueous solution of N-acetyl-L-cysteine at 1% (w/v), glutathione at 1% (w/v) and calcium lactate at 1% (w/v) **(CGLW)**, N-acetyl-L-cysteine at 1% (w/v), glutathione at 1% (w/v), calcium lactate at 1% (w/v) and DL-malic acid 2.5% (w/v) **(CGLW + MA)**, and distilled water **(W)** stored at 5ºC during 30 days. Reported values are the mean of two determination in duplicated (n = 4) ± standard deviation. *E. coli* O157:H7 counts in fresh-cut apples dipped in CGLW + MA resulted below detection limit (< 2 $\log_{10}$ CFU/g) after 3 days of storage and undetectable after a recovering step in buffer peptone water at 35-37ºc for 20 minutes.

Effect of the fresh-cut apples pH on pathogenic microorganisms

Fresh-cut apples pH was not affected by the addition of N-acetyl-L-cysteine, glutathione and calcium lactate to the dipping solution, since significant differences between pH of fresh-cut apples dipped in CGLW and those immersed in W were not found; however, when malic acid was

incorporated to the dipping solution, a decrease in the product pH was observed (Table 1). On the other hand, increases of pH of apple pieces through the storage time possibly due to the metabolic activity of the growing native microflora were detected for all treatment conditions, with maximum pH values of 4.29, 4.10 and 4.28, reached at 30 days of storage, for CGLW, CGLW + MA and W, respectively.

Table 1. pH values of fresh-cut "Fuji" apples inoculated with *L. monocytogenes*, *S.* Enteritidis or *E. coli* O157:H7 dipped in aqueous solutions containing quality stabilizing substances with or without malic acid and stored at 5ºc during 30 days.

Dipping condition	Storage time (days)	Fresh-cut apples pH*		
		Inoculated with *L. monocytogenes*	Inoculated with *S.* Enteritidis	Inoculated with *E. coli* O157:H7
CGLW	0	4.17 ± 0.02 Aa	4.19 ± 0.04 Aa	4.16 ± 0.04 Aa
	3	4.23 ± 0.01 Ab	4.21 ± 0.01 Aab	4.19 ± 001 Aab
	7	4.30 ± 0.08 Ad	4.23 ± 0.01 Abc	4.22 ± 0.05 Abc
	14	4.26 ± 0.06 Abc	4.22 ± 0.03 Aabc	4.24 ± 0.03 Ac
	21	4.27 ± 0.01 Acd	4.25 ± 0.04 Acd	4.28 ± 0.04 Ad
	30	4.30 ± 0.06 Ad	4.27 ± 0.05 Ad	4.29 ± 0.02 Ad
CGLW + MA	0	3.67 ± 0.02 Ba	3.85 ± 0.01 Ba	3.65 ± 0.03 Ba
	3	3.73 ± 0.03 Bb	3.88 ± 0.02 Bab	3.70 ± 0.01 Bb
	7	3.88 ± 0.04 Bc	3.93 ± 0.01 Bc	3.89 ± 0.02 Bc
	14	3.86 ± 0.13 Bc	3.93 ± 0.02 Bc	3.95 ± 0.02 Bd
	21	3.95 ± 0.06 Bd	3.91 ± 0.03 Bbc	3.90 ± 0.03 Bc
	30	4.08 ± 0.01 Be	4.02 ± 0.04 Bd	4.10 ± 0.04 Be
W	0	4.16 ± 0.06 Aa	4.17 ± 0.03 Aa	4.15 ± 0.03 Aa
	3	4.20 ± 0.01 Ab	4.21 ± 0.02 Ab	4.17 ± 0.02 Aa
	7	4.25 ± 0.04 Ac	4.24 ± 0.03 Abc	4.22 ± 0.03 Ab
	14	4.24 ± 0.02 Ac	4.25 ± 0.01 Ac	4.18 ± 0.01 Aa
	21	4.29 ± 0.03 Ad	4.23 ± 0.05 Abc	4.26 ± 0.05 Ac
	30	4.26 ± 0.03 Acd	4.25 ± 0.02 Ac	4.28 ± 0.02 Ac

*Means values of three determinations ± SD

CGLW Dipped in aqueous solution of N-acetyl-L-cysteine at 1% (w/v), glutathione at 1% (w/v) and calcium lactate at 1% (w/v).

CGLW + MA Dipped in aqueous solution of N-acetyl-L-cysteine at 1% (w/v), glutathione at 1% (w/v), calcium lactate at 1% (w/v) and malic acid at 2.5% (w/v).

W Dipped in sterile distilled water.

Different capital letters indicate significant differences ($p<0.05$) among dipping condition, whereas distinct lower-case letters show significant differences ($p<0.05$) among storage times

Nowadays, it is known that the pH-resistance of several microorganisms is depending not only on the kind of microorganism but also on its strain, specie or serotype. Populations of *L. monocytogenes*, *S.* Enteritidis and *E. coli* O157:H7 are able to grow from a minimum pH value of 4.3, 4.3 and 3.6, respectively (Meng and Doyle 1998; Lou and Yousef, 1999; Jay *et al.*, 2005). Below this pH value, inhibition of the microbial growth may occur. In our case, the initial pH of the fresh-cut apples treated with CGLW, CGLW+MA and W were below of those required for *L. monocytogenes* and *S.* Enteritidis growth, thus demonstrating why those microorganisms did not grow on apple pieces through the storage time. In contrast, pH of processed apple pieces was above that required for *E. coli* O157:H7 growth. Nevertheless, no growth was observed during the storage, indicating that other factors such as storage temperature and competitive native flora had a significant effect on the *E. coli* O157:H7 behavior on refrigerated fresh-cut apples. On the other hand, when fresh-cut apples were dipped in CGLW+MA a total inactivation of *L. monocytogenes* and *S.* Enteritidis was observed at the samples preparation day (t = 0 days). That same effect was detected for *E. coli* O157:H7 after 3 days of storage at 5ºC, demonstrating the already reported acid resistance of this microorganism (Meng et al., 2001) and indicating that a synergistic effect caused by storage temperature, competitive native flora and pH was responsible for the death of this microorganism. A decrease in cytoplasm pH of the cell caused by the internalization of the undissociated malic acid molecules could explain the inactivation of those microorganisms studied. Lou and Yousef, (1999) indicated that the antimicrobial action of organic acids is attributed to cytoplasm acidification, as well as to the specific antimicrobial effect of the particular anionic species. Those authors also indicated that undissociated organic acids can pass through the cell membrane, dissociate inside the cytoplasm, and interfere with metabolic processes of the microbial cell. In addition, Eswaranandan *et al.* (2004) demonstrated that organic acids with smaller molecular weight have a higher antimicrobial activity, suggesting that undissociated malic acid molecules (134.09 Dalton) may enter more easily into the bacterial cells than other acids such as citric (192.13 Dalton) and tartaric (150.09 Dalton).

Although safety of fresh-cut apples can be achieved for at least 30 days of storage by incorporating malic acid at 2.5% into the dipping solutions, previous studies have limited the shelf life of fresh-cut apples immersed into a solution of 1% N-acetyl-L-cysteine, 1% glutathione, 1% calcium lactate and 2.5% malic acid to 14 days, mostly due to changes in physicochemical parameters such as firmness, color and headspace gas

composition (Raybaudi-Massilia et al., 2007). Nonetheless, Raybaudi-Massilia et al., (2007) demonstrated that the addition of malic acid and stabilizing substances to fresh-cut apples by dipping treatments enhanced its taste, resulting preferred by the panelists those fresh-cut apples treated with CGLW + MA in comparison with those dipped in W.

CONCLUSIONS

A dipping solution containing 1% N-acetyl-L-cysteine, 1% glutathione, 1% calcium lactate and 2.5% malic acid was effective to achieve more than 5 log reductions of *L. monocytogenes* and *S.* Enteritidis at the samples preparation day (t = 0 days) and after 3 days of refrigerated storage for *E. coli* O157:H7, demonstrating that the safety of fresh-cut apples could be assure using that dipping treatment. It has been also demonstrated that, among the pathogenic microorganisms analyzed, *E. coli* O157:H7 is the most resistant to the dipping treatments; therefore, this pathogen can be considered as target microorganism in this kind of product.

The stabilizing substances used in these experiments showed to possess antimicrobial activity against the pathogenic microorganisms inoculated in fresh-cut apples; however, when malic acid was added to the dipping solution a greatest bactericidal effect on these microorganisms was observed.

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Shelf-life extension of fresh-cut Fuji apples at different ripeness stages using natural substances

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ABSTRACT

Shelf-life extension of fresh-cut "Fuji" apple at two stages of ripeness (partially ripe and ripe) using natural substances was evaluated. Cylinders of fresh-cut "Fuji" apples were immersed for 1 min in (a) an aqueous solution of N-acetyl-L-cysteine at 1% w/v, glutathione at 1% w/v and calcium lactate at 1% w/v (CGLW), (b) CGLW and D-L -malic acid at 2.5% w/v (CGLW + MA) or (c) sterile distilled water (W). Gas production, firmness, color and behavior of native flora (mesophilic bacteria, psychrophilic bacteria, yeasts and mould) were studied weekly over 30 days. Sensory evaluation of fresh-cut apples was carried out at 0 and 15 days during storage. Statistically significant differences ($p < 0.05$) were found in ethylene and ethanol production between fresh-cut partially ripe and ripe apples, respectively reaching values of ethylene of 8.92 and 134.11µl $.l^{-1}$ and ethanol of 28.73 and 59.39 µl $.l^{-1}$ at 30 days of storage. The firmness of fresh-cut apples was significantly different ($p < 0.05$) between partially ripe (initial values of 12.4 ± 0.8 N) and ripe apples (initial values of 8.3 ± 1.0 N), but did not differ throughout the storage time, nor among dipping conditions. An important reduction in lightness ($p < 0.05$) was observed throughout storage in the fresh-cut apples with all dipping conditions. However, an influence of the ripeness stage on microbiological stability was not detected. A reduction in growth rate and an increase in the lag phase of mesophilic, psychrophilic, yeasts and mold were found in fresh-cut apples dipped in CGLW + MA, which gave as a result an extension of 13 days over the microbiological stability of fresh-cut apples immersed in W (10.1 days). Thus, the use of a combination of CGLW + MA might be a good low cost alternative for the fresh-cut industry since it can offer better maintenance of the physicochemical characteristics and microbiological stability of fresh-cut apples, ensuring a shelf-life of, at least, 14 days.

Keywords: apple; shelf-life; ripeness; malic acid; N-acetyl-L-cysteine; glutathione; calcium lactate.

INTRODUCTION

In recent years a rapid expansion in the sale of pre-packed/pre-cut fresh fruit and vegetables is taking place because of the advantages offered by those commodities to consumers, such as freshness, convenience and low calorific content. However, as a result of the active metabolism of the plant tissues, and damage during peeling, grating, shredding and the exposure of their cut surfaces to external factors, the produce changes from having a relative stability with a shelf-life of several weeks or months to a perishable product with a very short shelf-life (Ahvenainen, 1996; Lanciotti et al. 1999). For those reasons, achieving microbiologically safe products with sensory and nutritional fresh-like characteristics is still a challenge, in spite of the research efforts already made.

The deterioration of lightly processed fruit is a complex process concerning both physicochemical and biochemical modifications and microbial spoilage. Injury stress caused by processing results in cellular decompartmentalization or delocalization of enzymes and substrates which leads to various type of biochemical deterioration such as browning, off-flavors, and texture breakdown. Moreover, peeling and cutting facilitate primary infection of the plant tissues by epiphytic and phytopathogenic microorganisms (Varoquaux and Wiley, 1994).

Enzymes and substrates are normally located in different cellular compartments and their transfer is actively regulated. Processing results in destruction of surface cells and injury stress of underlying tissues. Enzymatic reactions cause sensory deterioration such as off-flavor, discoloration, and loss of firmness (Varoquaux and Wiley, 1994). Enzymatic browning of minimally processed apples is a major problem affecting processing of the fruit, polyphenol oxidase being the most important enzyme involved. Enzymatic browning requires the presence of four components: oxygen, an oxidizing enzyme, copper and a suitable substrate. To prevent browning, at least one of these components must be removed from the system (Ahvenainen, 1996). Browning and its control have been extensively studied and reported in foods including fruit and vegetables (Iyengar and McEvily, 1992; McEvily and Iyengar, 1992; Ahvenainen, 1996; Friedman, 1996; Laurila et al., 1998). Traditionally, sulphites have been used to prevent browning in fruit and vegetables; however their use can have negative health effects, such as with asthma (Ahvenainen, 1996). Therefore, various approaches to control the extent of browning have been tried, such as the use of restricted O_2 atmosphere conditions with low-temperature storage to prevent enzymatic browning of

apple pieces. However, this approach is not sufficient to attain browning stability because the high phenolic content of the fruit diminishes the effectiveness of elevated CO_2 atmospheres in preventing browning of fresh-cut apples; it has been concluded that reduction of O_2 levels to negligible levels is necessary to inhibit polyphenol oxidase (PPO) mediated browning of many fresh-cut fruit products (Buta et al. 1999). Other approach proposed are the use of browning inhibitors with several different types of biochemical functions alone or in combination with modified atmospheres and low temperature storage. Reducing agents such as citric acid, ascorbic acid, isoascorbic acid, sodium erythorbate and 4-hexylresorcinol (Ahvenainen, 1996; Sapers and Miller, 1998; Buta et al. 1999; Dong et al., 2000; Soliva-Fortuny et al. 2001; 2002), as well as sulphydryl (SH)-containing amino acids and peptides such as cysteine and glutathione (Molnar-Perl and Friedman, 1990; Richard et al., 1991; 1992; Buta, et al., 1999; Gorny et al., 2002; Rojas-Graü et al., 2006; Oms-Oliu et al., 2006), have been used to prevent the browning of fresh-cut apples, pears and potatoes. Calcium salts, particularly calcium chloride and lactate, are used generally in combination with those browning inhibitors as firmness agents in a wide variety of whole, peeled, and fresh-cut fruit and vegetables.

Minimal processing may increase microbial spoilage of fruit through transferring skin microflora to fruit flesh, where microorganisms can grow rapidly upon exposure to nutrients (Corbo et al., 2004). Fungi and mesophilic bacteria are the principal flora present on fruit, mainly because of their pH. However the incidence of other microorganisms as parasites, viruses and food-borne pathogenic bacteria, such as *Listeria monocytogenes*, *Salmonella* serovars and some *E. coli* strains, has also been reported (Harris *et al.* 2003; Lanciotti *et al.* 2003; Eswaranandam *et al.* 2004). In fact, the number of documented outbreaks of human infections associated with consumption of raw and minimally processed fruit and vegetables has considerably increased during the past decades (Lanciotti et al., 2003).

The use of natural antimicrobial compounds, such as organic acids, is a good practise for the food industry because they can prevent microorganism growth and additionally avoid browning. The main acid present in apple is malic and its concentration varies widely among apples varieties (Eisele and Drake 2005).

The main objective of this study was to extend the shelf-life of fresh-cut "Fuji" apples at different ripeness stages using of N-acetyl-L-cysteine, glutathione, calcium lactate **a**nd D-L -malic acid.

MATERIALS AND METHODS

Materials

"Fuji" apples **(***Malus domestica* Borkh), partially ripe (commercial ripeness stage) and ripe (advanced ripeness stage) apples were provided by ACTEL, Lleida, Spain. N-acetyl-L-cysteine with 98% degree purity (Acros Organics, New Jersey, USA) and reduced glutathione with 98% degree purity (Acros Organics, New Jersey, USA) were used as antibrowning substances, calcium lactate pent-hydrate, extra pure (Scharlau Chemie S. A. Barcelona, Spain) was applied as an anti-softening agent and D-L -malic acid, extra pure (Scharlau Chemie S. A. Barcelona, Spain), was used as an antimicrobial.

Flesh apple characterization

Characterization of the apples was made following the official methods for fruit juices and other vegetables and derivatives (B.O.E., 1988). Titrable acidity, pH (Crison 2001 pH-meter; Crison Instruments S. A., Barcelona, Spain), soluble solids (%) content (Atago RX-1000 refractometer; Atago Company Ltd, Japan), color (Minolta CR-400 Chroma Meter; Konica Minolta Sensing, INC. Osaka, Japan) and firmness (TA-TX2 Texture Analyzer; Stable Micro Systems LTD. Surrey, England) were the evaluated parameters (Table 1).

Table 1. Physicochemical properties of fresh-cut partially ripe and ripe "Fuji" apple.

Parameters		Partially ripe	Ripe
pH		4.20 ± 0.01*	4.37 ± 0.01*
Soluble Solids (%)		12.4 ± 0.1*	13.50 ± 0.17*
Total acidity (malic ac. g/100 ml)		0.421 ± 0.013*	0.233 ± 0.006*
Color	**L***	75.0 ± 1.1**	72.53 ± 0.30**
	a*	- 1.40 ± 0.27**	- 1.96 ± 0.23**
	b*	22.9 ± 1.1**	22.5 ± 0.5**
Firmness (N)		13.8 ± 1.3**	7.2 ± 0.6**

*Mean of three analysis ± standard deviation
** Mean of ten analysis ± standard deviation

Dipping solutions

Two aqueous solutions of N-acetyl-L-cysteine at 1% w/v, reduced glutathione at 1% w/v, calcium lactate pent-hydrate at 1% w/v in sterile distilled water (CGLW), and N-acetyl-L-cysteine at 1% w/v, reduced glutathione at 1% w/v, calcium lactate pent-hydrate at 1% w/v, D-L -malic acid at 2.5% w/v in sterile distilled water (CGLW+MA), were prepared. Sterile distilled water (W) was used as the control treatment. The concentrations of N-acetyl-L-cysteine, glutathione and calcium lactate were chosen in accordance with previous studies (Gorny et al., 2002; Oms-Oliu et al., 2006; Rojas-Graü et al., 2006), whereas the D-L malic acid concentration was selected according to preliminary trials performed in the laboratory (unpublished data).

Sample preparation

Partially ripe and ripe **a**pples were sanitized by immersing the fruit in 300 μl $.l^{-1}$ chlorine for 5 min; then washed with potable water and finally dried with absorbent paper. All cutting boards, utensils, and holding containers were also sanitized with 300 μl $.l^{-1}$ chlorine. The cleaned fruit were cut in the extremes with a knife, and then with a cylindric hollow instrument of stainless steel into pieces of 1.4 cm diameter x 2.0 cm height (approximately 6 cylinders per apple). The cut fruit were divided into three parts and then dipped for 1 min (Rojas-Graü et al., 2006) in three different solutions with a ratio fruit: solution = 1:2 and constant agitation using a magnetic stirrer. After draining the excess of liquid, 10 cylinders with a total weight of 50 g were placed on polypropylene trays (173 x 129 x 35 mm). The trays were sealed using a thermo-sealing machine, ILPRA Food Pack Basic V/6 (Ilpra S. CP. Vigevono, Italy). The permeability of the plastic used for sealing was: 142.86 $fmol.s^{-1}.m^{-2}.kPa^{-1}$ for water vapor at 38ºC, 90% H.R., 52.38 $fmol.s^{-1}.m^{-2}.kPa^{-1}$ for O_2 at 23ºC, 0% H.R. and 2.38 $fmol.s^{-1}.m^{-2}.kPa^{-1}$ for CO_2 at 23ºC, 0% H.R. A total of 20 trays for each dipping condition and ripeness stage, were prepared and stored at 5ºC. A pair of those trays was randomly taken at 0, 7, 14, 21 and 30 days for physicochemical determinations and the other two trays for microbiological analysis.

Headspace gas evaluation

The atmosphere of each tray was analyzed in duplicate by gas chromatography. A Micro-GP CP 2002 gas analyzer (Chrompack International, Middelburg, Netherlands) equipped with a thermal conductivity detector was used. A little adhesive septum was stuck to the plastic that seals the tray to reinforce the place where the gas sample is taken, thus avoiding leakage of gas. A sample of 1.7 ml was automatically withdrawn from the headspace atmosphere. Portions of 0.25 and 0.33 ml were injected for O_2 and CO_2 determination, respectively. The O_2 content was analyzed with a CP-Molsieve 5Å packed column (Chrompack International, Middelburg, Netherlands) (4m x 0.32mm, df =10mm) at 60ºC and 100 kPa. A Pora-PLOT Q column (Chrompack International, Middelburg, Netherlands) (10m x 0.32mm, df =10mm) was held at 70ºC and 200 kPa for CO_2, ethylene, and ethanol quantification. A pair of trays randomly taken was analyzed for each dipping condition and ripeness stage at 0, 7, 14, 21 and 30 days.

Color evaluation

The color determination was made weekly over 30 days of storage at 5ºC in a pair of trays randomly chosen by dipping condition and ripeness stage. A tri-stimulus Minolta CR-400 Chroma Meter (Konica Minolta Sensing, INC. Osaka, Japan) using the illuminant D75 and observation angle of 10º, which had been calibrated with a standard white plate (Y = 94.00, x = 0.3158, y = 0.3322) was employed. Three readings of L* (Lightness), a* (green chromaticity) and b* (yellow chromaticity) coordinates were recorded for each apple cylinder. Ten cylinders were analyzed by each pair of trays. Thus, the reported values are the mean of thirty determinations ± S.D. Hue angle hº was also calculated.

Firmness evaluation

The firmness of the flesh tissue was measured weekly during 30 days of storage at 5ºC, in a pair of trays randomly taken for each dipping condition and ripeness stage. The firmness evaluation was carried out by determination of the maximal strength of penetration using a TA-TX2 Texture Analyzer (Stable Micro Systems LTD. Surrey, England) at room temperature and the following conditions: pre-test speed: 2 mm/s, test

speed: 5.0 mm/s, post-test speed: 5.0 mm/s and penetration distance: 10 mm. The resistance of the penetration was measured as the strength necessary for a cylindrical probe of 4 mm of diameter and plane basis to penetrate a cylindrical sample of apple flesh of 2.00 cm height. Ten cylinders of fresh-cut apples were measured for each pair of trays.

Microbiological analysis

Mesophilic, psychrophilic, yeast and mould counts were carried out weekly over 30 days in duplicate for each single tray. A pair of trays for each dipping condition and ripeness stage was analyzed. The counts were reported as Log_{10}CFU/g.

Mesophilic and psychrophilic bacteria counts were made according to the ISO 4833:1991 guideline using Plate Count Agar (PCA) Biokar Diagnostics, Beauvais, France and the pour plate method. The plates of psychrophilic bacteria were incubated at 5ºC for 10-14 days, whereas mesophilic bacteria were incubated at 35ºC for 48 h.

Yeast and mould counts were made according to the ISO 7954:1987 guideline using Chloramphenicol Glucose Agar (CGA) Biokar Diagnostics, Beauvais, France, and the spread plate method. The plates were incubated at room temperature for 3-5 days.

Sensory evaluation

Partially ripe fresh-cut apples dipped in the different aqueous solutions were used to carry out a sensory analysis in a similar way to that of Saftner et al. (2005). Thirty volunteers, who like and eat apples frequently, evaluated apple cylinders dipped in CGLW, CGLW + MA or W prepared the same day of the test (t = 0) and also those stored at 5ºC for 15 days. A total of 6 samples labeled with 3 digit code numbers were randomly provided to the panelists, who rated acceptability of odor, color, taste, acidity and firmness characteristics on structured 10 cm scales labeled from “extremely unpleasant” to “extremely pleasant”. That information was converted to scores from 0 to 10, respectively.

Data analysis

Statistical analysis was performed using Statgraphics Centurion XV version 15.1.02. A multifactor ANOVA with posterior Multiple Range

Test was used to find significant differences among the effects of ripeness stages, storage time and dipping condition on gas production, firmness, color, microbiological count and sensory evaluation profile.

Mesophilic and psychrophilic bacteria, yeast and mould data, expressed as Log_{10} CFU/g were modeled according to the Gompertz equation modified by Zwietering et al. (1990) (Equation 1):

$$Y = k + A \cdot exp\{-exp[(\mu_{max} \cdot 2.7182 / A)(\lambda - t) + 1]\} \quad (1)$$

where Y is the count of microorganisms (log_{10} CFU/g) for a given time, k is the microorganism initial count estimated by the model (log_{10} CFU/g), A is the maximum microorganism growth attained at the stationary phase (log_{10} CFU/g), μ_{max} is the maximal growth rate [Δ log_{10} (CFU/g)/day], λ is the lag time (days) and t is the storage time (days).

Shelf-life of fresh-cut apples at two ripeness stages was calculated from equation (2) obtained from Gompertz modified by Zwietering et al. (1990) (Equation 1). This equation considers as the maximum limit of mesophilic aerobic total count at the expiry date the value allowed by the Spanish regulations for hygienic processing, distribution and commerce of prepared meals (B.O.E, 2001) (10^7 CFU/g).

$$SL = \lambda - \frac{A\left\{ln\left[-ln\left(\frac{log(10^7) - k}{A}\right)\right] - 1\right\}}{\mu_{max} \cdot 2.7182} \quad (2)$$

RESULTS AND DISCUSSION

Headspace gases

The change of the package headspace gas composition of "Fuji" apple tissue at both partially ripe and ripe stages was determined weekly. Fresh-cut partially ripe apple tissue dipped in W initially consumed more oxygen than ripe apple tissue, decreasing the concentrations of oxygen to 4.23 and 9.08 kPa, respectively, after 14 days of storage (Fig. 1). However, oxygen levels tended to be stabilized after 14 days of storage when partially

ripe apples were used but continued to decrease when ripe apples were employed. Likewise, carbon dioxide production was higher in fresh-cut partially ripe apple pieces than ripe apple pieces dipped in W, the concentrations at 14 days being 14.26 and 9.95 kPa, respectively. Statistically significant differences ($p<0.05$) in gas composition between the two ripeness stages and storage times were found through a multifactor ANOVA with posterior Multiple Range Test, showing that partially ripe cut apple had the highest oxygen consumption and the highest carbon dioxide production (Fig. 1 and 2).

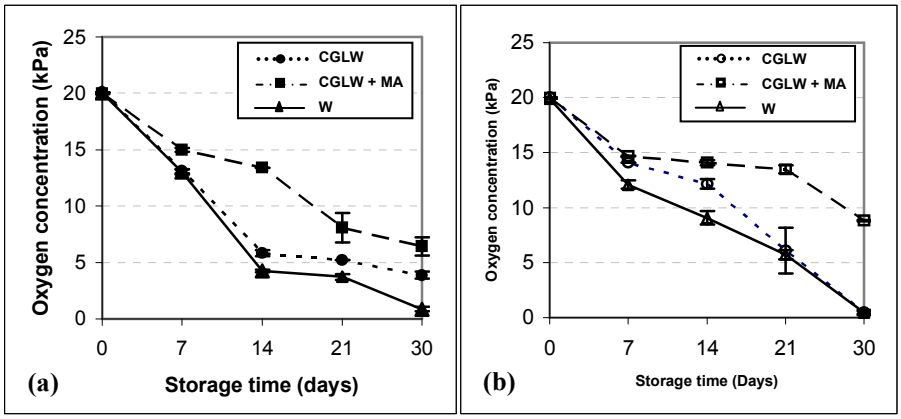


Figure 1.- Influence of ripeness stage on the oxygen concentration (kPa) in fresh-cut "Fuji" apple partially ripe (a) or ripe (b) dipped in aqueous solutions of N-Acetyl-L-cysteine at 1% (w/v), glutathione at 1% (w/v) and calcium lactate at 1% (w/v) (CGLW), N-Acetyl-L-cysteine at 1% (w/v), glutathione at 1% (w/v), calcium lactate at 1% (w/v) and DL-malic acid 2.5% (w/v) (CGLW + MA) and distilled water as control (W) packaged in air and stored at 5ºC during 30 days. The values are mean of 4 determinations ± S.D.

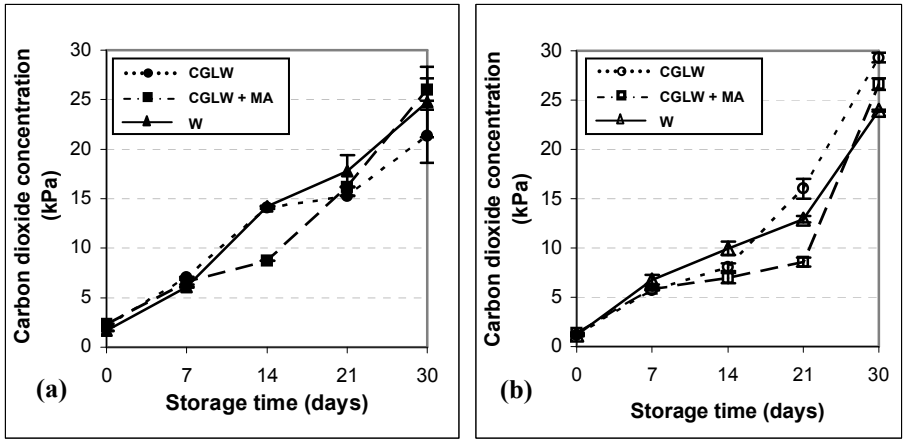


Figure 2.- Influence of ripeness stage on the carbon dioxide concentration (kPa) in fresh-cut "Fuji" apple partially ripe (a) or ripe (b) dipped in aqueous solutions of N-Acetyl-L-cysteine at 1% (w/v), glutathione at 1% (w/v) and calcium lactate at 1% (w/v) (CGLW), N-Acetyl-L-cysteine at 1% (w/v), glutathione at 1% (w/v), calcium lactate at 1% (w/v) and DL-malic acid 2.5% (w/v) (CGLW + MA) and distilled water as control (W) packaged in air and stored at 5ºC during 30 days. The values are mean of 4 determinations ± S.D.

In addition, smaller amounts of ethylene (Fig. 3a) and ethanol (Fig. 4a) were found inside the packages of partially ripe cut apple in comparison with those of ripe apple pieces (Fig. 3b and 4b), reaching concentrations up to 8.92 and 28.73 μl $.l^{-1}$ and 134.11 and 59.39 μl $.l^{-1}$ of ethylene and ethanol, respectively, at 30 days of storage using W as dipping solution. This confirms that ethylene production increases since apple pieces are living tissues that continue ripening after cutting, following a classic behaviour of climacteric fruit (Wills et al., 1998). A comparable change in ethylene and ethanol concentrations in minimally processed apple was reported by Soliva-Fortuny et al. (2002), who evaluated the effects of ripeness stages on the storage atmosphere, color, and textural properties of minimally processed apple slices. Low concentrations of ethylene in the package headspace of fresh-cut partially ripe apples are indicative of reduced wounding stress, indicating that ethylene - mediated wounding

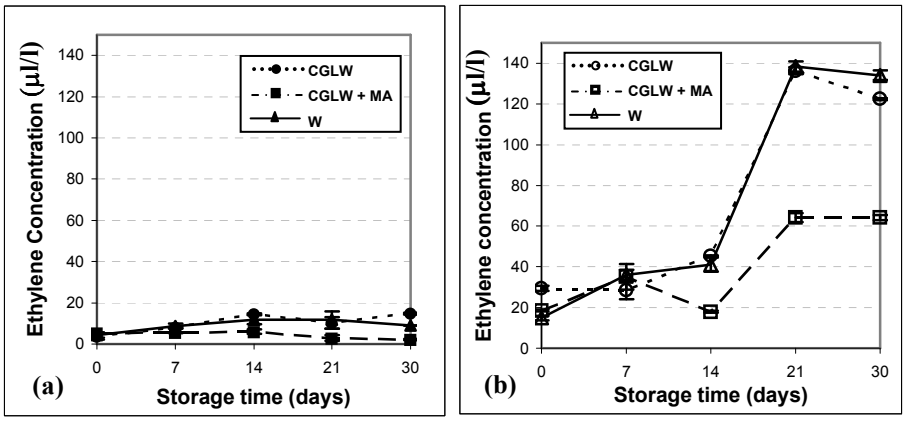


Figure 3.- Influence of ripeness stage on the ethylene concentration (µl/l) in fresh-cut "Fuji" apple partially ripe (a) or ripe (b) dipped in aqueous solutions of N-Acetyl-L-cysteine at 1% (w/v), glutathione at 1% (w/v) and calcium lactate at 1% (w/v) (CGLW), N-Acetyl-L-cysteine at 1% (w/v), glutathione at 1% (w/v), calcium lactate at 1% (w/v) and DL-malic acid 2.5% (w/v) (CGLW + MA) and distilled water as control (W) packaged in air and stored at 5ºC during 30 days. The values are mean of 4 determinations ± S.D.

response to cutting operations is substantially reduced when apples are processed at a partially ripe stage. Low oxygen and high carbon dioxide environments could partially explain the inhibition of ethylene formation in fresh-cut partially ripe apples. It is widely known that oxygen participates in the conversion of 1-amino-cyclopropane-1-caboxylic acid (ACC) to ethylene (Yang, 1981). However oxygen levels reached throughout storage did not justify the low production of fermentative anaerobic respiratory metabolites in fresh-cut partially ripe apples. On the other hand, a relationship among the oxygen concentration and the ethylene and ethanol production was observed in fresh-cut ripe apples.

Dipping treatments significantly ($p<0.05$) affected oxygen concentration in the package headspace. Thus, fresh-cut apples dipped in a CGLW+MA solution consumed less oxygen throughout storage than apples dipped in CGLW or W. Consistently, in CGLW + MA treated fresh-cut

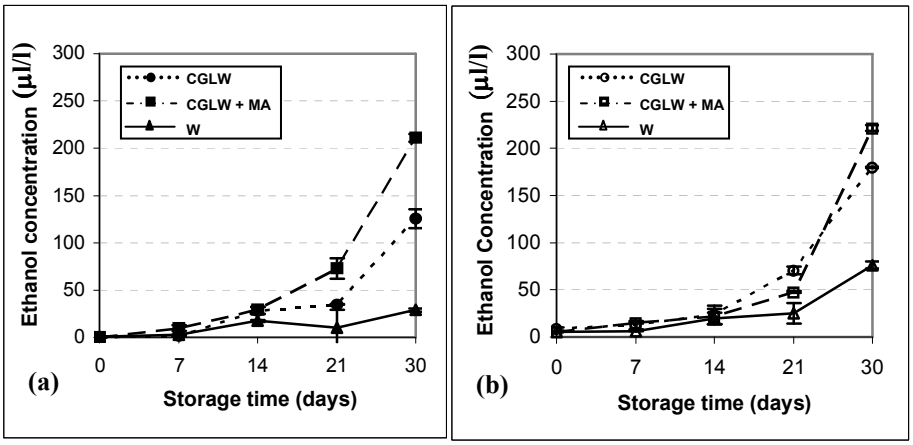


Figure 4.- Influence of ripeness stage on the ethanol concentration (µl/l) in fresh-cut "Fuji" apple partially ripe (a) or ripe (b) dipped in aqueous solutions of N-Acetyl-L-cysteine at 1% (w/v), glutathione at 1% (w/v) and calcium lactate at 1% (w/v) (CGLW), N-Acetyl-L-cysteine at 1% (w/v), glutathione at 1% (w/v), calcium lactate at 1% (w/v) and DL-malic acid 2.5% (w/v) (CGLW + MA) and distilled water as control (W) packaged in air and stored at 5ºC during 30 days. The values are mean of 4 determinations ± SD.

apples, carbon dioxide production was considerably slowed down during the first 14 days of storage irrespective of the apple ripeness. These changes in the atmosphere composition could be attributed in part to the effect of malic acid against microorganisms. Nevertheless, addition of malic acid in the dipping formulation also appeared to control ethylene production in fresh-cut ripe apples (Fig. 3), since lower concentrations of this gas were found in comparison with fresh-cut apples dipped in CGLW or W solutions. These last dipping conditions could not prevent a dramatic increase in ethylene levels inside the packages, especially beyond 14 days storage.

On the other hand, the ethanol production increased after 14 days of storage in all the assayed conditions, showing a significant increase in fresh-cut apple dipped in CGLW and CGLW + MA in comparison with control dipping treatment (W).

The results found in this study demonstrate that the use of a combination of natural substances as CGLW + MA could reduce the deterioration of fresh-cut apples stored at refrigeration temperatures, since the oxygen consumption and the carbon dioxide and ethylene production are reduced during storage time.

Firmness of fresh-cut apples

The firmness of the cut apple differed considerably between ripeness stages (Table 2) showing initial values of 12.4 ± 0.8 N and 8.3 ± 1.0 N for the partially ripe and ripe cut apple dipped in W respectively. Texture degradation has been closely correlated to ripening processes. Firmness changes are supposed to be sparked off by the action of pectin enzymes, especially polygalacturonases (Knee 1973). During ripening, there is a rapid synthesis of the enzyme and a subsequent release of soluble Ca, which would explain the higher softening experienced by ripe fruit. Soliva-Fortuny et al. (2002) also reported important differences in firmness among ripeness conditions in "Golden Delicious" apple. However significant effects ($p>0.05$) of storage time and dipping conditions were not detected (Table 2). Soliva-Fortuny et al. (2002) found different results in "Golden Delicious" fresh-cut apples showing that firmness differed considerably among ripeness conditions. Those authors reported that mature-green apples best maintained their initial firmness (8.2 N), followed by partially ripe (6.3 N) and ripe apples (3.9 N). An important difference can be noted after comparing the initial values obtained by Soliva-Fortuny et al. (2002) with ours, indicating that apple variety is a significant factor influencing the change of firmness through storage time.

Color changes in fresh-cut apples

Ripeness stage and storage time had a significant effect ($p<0.05$) on the lightness (L*) of fresh-cut apples (Fig. 5). Lower values of lightness were observed for ripe tissue (Fig. 5b) than for partially ripe tissue (Fig. 5a). A significant reduction ($p<0.05$) in lightness throughout storage was also observed for all dipping conditions. A similar reduction of lightness throughout storage time was reported by Soliva-Fortuny et al. (2001, 2002) in "Golden Delicious" fresh-cut apple and Oms-Oliu et al. (2006) in fresh-cut "Flor de invierno" pear. Large changes observed in fresh-cut ripe apple color could be explained, firstly, by a greater decompartmentalization of oxidases, as polyphenol oxidase and peroxidase, and their substrates.

Table 2. Firmness of fresh-cut partially ripe and ripe "Fuji" apple dipped in aqueous solutions of natural substances and stored at 5ºC for 30 days.

Dipping condition	Time (days)	Firmness (N)*	
		Partially ripe apple	Ripe apple
CGLW	0	12.8 ± 1.1	7.4 ± 0.7
	7	11.6 ± 0.6	8.0 ± 0.9
	14	13.1 ± 0.7	7.4 ± 0.4
	21	13.5 ± 1.0	9.0 ± 0.6
	30	13.6 ± 1.5	7.3 ± 0.4
CGLW + MA	0	11.6 ± 1.2	8.3 ± 1.1
	7	12.99 ± 0.29	8.4 ± 0.8
	14	11.9 ± 1.0	7.6 ± 1.2
	21	13.08 ± 0.29	6.5 ± 1.4
	30	10.8 ± 1.2	7.7 ± 0.5
W	0	12.4 ± 0.8	8.3 ± 1.0
	7	11.6 ± 0.3	6.3 ± 0.7
	14	10.5 ± 1.0	7.2 ± 1.4
	21	13 ± 3	8.06 ± 0.05
	30	11.8 ± 2.9	8.4 ± 2.1

* Mean of 10 values ± SD
CGLW Dipped in aqueous solution of N-acetyl-L-cysteine at 1% (w/v), glutathione at 1% (w/v) and calcium lactate at 1% (w/v)
CGLW + MA Dipped in CGLW + malic acid at 2.5% (w/v)
W Dipped in sterile distilled water
Significant differences ($p<0.05$) among apple ripeness stages were found, but not among dipping conditions and storage times.

Secondly, a higher ethylene production may induce a rise in phenylalanine ammonia lyase activity, which would increase the synthesis of phenolic compounds, thus resulting in increased browning susceptibility in ripe tissues exposed to adverse conditions. Likewise, L* values was also dipping conditions in fresh-cut partially ripe tissue, where lower values were observed for fresh-cut apples dipped in CGLW + MA in comparison with fresh-cut apples immersed in CGLW and W, whereas in fresh-cut ripe apple significant differences were not found. Dipping condition and storage time also significantly ($p<0.05$) affected hue angle (hº) values. Fresh-cut apples dipped in CGLW + MA and CGLW maintained higher hº values than those immersed in W (Fig. 6). On the other hand, significant reductions ($p<0.05$) of hº values were observed especially after 14 days of

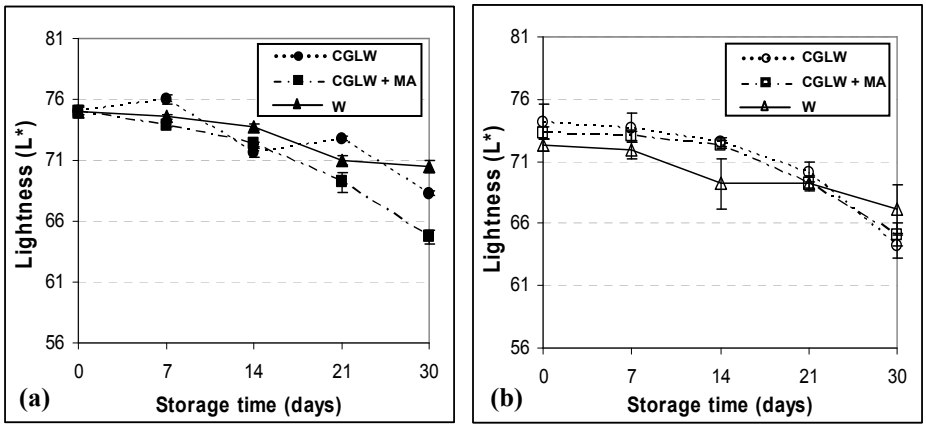


Figure 5.- Influence of ripeness stage on the lightness (L*) in fresh-cut "Fuji" apple partially ripe (a) or ripe (b) dipped in aqueous solutions of N-Acetyl-L-cysteine at 1% (w/v), glutathione at 1% (w/v) and calcium lactate at 1% (w/v) (CGLW), N-Acetyl-L-cysteine at 1% (w/v), glutathione at 1% (w/v), calcium lactate at 1% (w/v) and DL-malic acid 2.5% (w/v) (CGLW + MA) and distilled water as control (W) packaged in air and stored at 5ºC during 30 days. The values are mean of 30 determinations ± S.D.

storage. These results demonstrate that the natural substances selected in this study are adequate to prevent browning of cut apples and maintain the fresh-cut apple color for 14 days. Molnar-Perl and Friedman, (1990) and Rojas-Graü et al., (2006) found similar results when evaluating the effectiveness of different substances to prevent apple browning, indicating that N-acetyl-L-cysteine and glutathione are good antibrowning substances. Molnar-Perl and Friedman, (1990) and Richard-Forget et al., (1991; 1992) indicated that cysteine, N-acetyl-L-cysteine and reduced glutathione can prevent browning by competitive reaction with polyphenol oxidase, reacting with the intermediate quinones to form stable colorless compounds. In addition we found that the incorporation of malic acid in the dipping solution helps to maintain a better color of the fresh-cut apples during storage time, thus inducing its antioxidant activity. Meyer et al. (2002) indicated that organic acids as citric, tartaric and malic prevent the browning of food by acting as metal chelators.

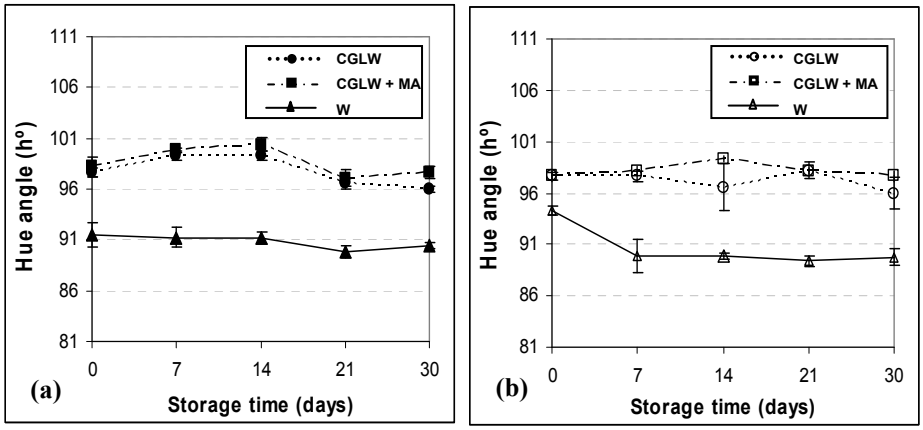


Figure 6.- Influence of ripeness stage on the hue angle (hº) in fresh-cut "Fuji" apple partially ripe (a) or ripe (b) dipped in aqueous solutions of N-Acetyl-L-cysteine at 1% (w/v), glutathione at 1% (w/v) and calcium lactate at 1% (w/v) (CGLW), N-Acetyl-L-cysteine at 1% (w/v), glutathione at 1% (w/v), calcium lactate at 1% (w/v) and DL-malic acid 2.5% (w/v) (CGLW + MA) and distilled water as control (W) packaged in air and stored at 5ºC during 30 days. The values are mean of 30 determinations ± S.D.

Microbial stability of fresh-cut apples

Both ripeness stages of fresh-cut apples showed no significant differences ($p < 0.05$) in microbial stability, since mesophilic and psychrophilic bacteria, yeasts and mould showed similar behavior for the two ripeness stages; mean values were therefore calculated. These results are consistent with the fact that microbial load does not depend on the ripeness stage, but on the quality of the raw materials and processing practices.

A significant increase ($p<0.05$) in microorganism populations in fresh-cut apples was observed throughout storage time for each dipping treatment (Fig. 7). Microbial populations of mesophilic bacteria (Fig. 7a), psychrophilic bacteria (Fig. 7b) or yeasts and mould (Fig. 7c) ranged approximately from 2-2.5 $\log_{10}$ CFU/g at day 0 to over 7.5 $\log_{10}$ CFU/g at day 30 of storage. Similar behavior was reported by different authors, such as Soliva-Fortuny et al. (2004) studying microbiological and biochemical stability of fresh-cut "Golden Delicious" apples preserved by modified atmosphere packaging, Corbo et al. (2004) evaluating the effect of temperature on shelf-life and microbial populations of lightly processed "Cactus" pear fruit, or Sinigaglia et al. (2003) modelling shelf-life of ready-to-eat coconut. However, mesophilic bacteria, psychrophilic bacteria, and yeast and mould development depended on the dipping condition. The highest microbial population during storage corresponded to fresh-cut apples dipped in W, whereas apple pieces dipped in CGLW + MA had the lowest counts. The microbial load of fresh-cut apples dipped in CGLW remained between the counts of fresh-cut apples dipped in W and CGLW+MA (Fig. 7).

The behavior of native microflora in fresh-cut apples was modelled by the Gompertz equation modified by Zwietering et al. (1990) (Eq. 1). This equation was applied considering the experimental data all together, because no differences were found between fresh-cut apples obtained from partially ripe and ripe apples in this regard. The model satisfactorily fitted the experimental data since the coefficient of determination ranged from 94.9 to 99.5 %. Table 3 shows mean values of Gompertz parameters that described the microbial growth of native microflora in the two ripeness stages.

In general, the addition of natural substances into dipping solutions delayed the proliferation of the studied populations. That effect was particularly intensified when malic acid was incorporated to dipping solutions, showing higher values of the lag time (λ) for mesophilic (2.5 ± 2.0 days) and psychrophilic (1.4 ± 1.1 days) populations in fresh-cut apples dipped in CGLW + MA than those found in fresh-cut apples immersed in CGLW (0.19 ± 0.08 days and 0.22 ± 0.12 days) and W (2.2 ± 1.6 days and 0.01 ± 0.04 days) (Table 3). On the other hand, more rapid microbial growth from the first day of storage as well as a rapid attainment of a stationary phase were observed for the populations in fresh-cut apples dipped in W compared with those dipped in CGLW or CGLW+MA, since the maximal growth rate, $\mu_{max,}$ was reduced by the incorporation of

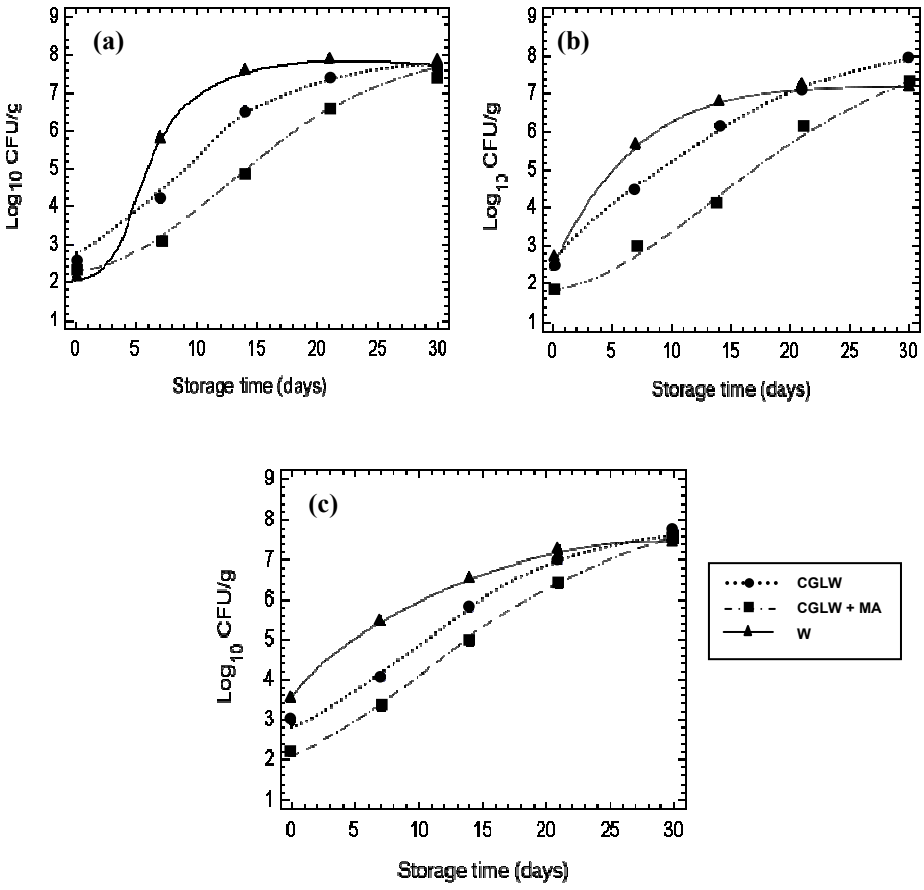


Figure 7.- Influence of the dipping condition and storage time on mesophilic bacteria (a), psychrophilic bacteria (b), yeast and mould (c) growth (Log_{10} CFU/g) in fresh-cut "Fuji" apple dipped in aqueous solutions of N-Acetyl-L-cysteine at 1% (w/v), glutathione at 1% (w/v) and calcium lactate at 1% (w/v) (CGLW), N-Acetyl-L-cysteine at 1% (w/v), glutathione at 1% (w/v), calcium lactate at 1% (w/v) and DL-malic acid 2.5% (w/v) (CGLW + MA) and distilled water as control (W) packaged in air and stored at 5ºC during 30 days. The values are mean of 8 determinations of the two ripeness stages.

Table 3. Mean values of the Gompertz parameters that describe the growth of mesophilic, psychrophilic, yeast and mould in fresh-cut "Fuji" apple dipped in aqueous solutions of natural substances and stored at 5ºC for 30 days.

Population	Dipping condition	R^2	MSE	Gompertz parameters*			
				k	A	μ_{max}	λ
Mesophilic	CGLW	97.2	0.16	2.0 ± 1.1	5.45 ± 0.26	0.28 ± 0.05	0.19 ± 0.08
	CGLW + MA	99.5	0.03	2.02 ± 0.24	5.4 ± 0.5	0.271 ± 0.023	2.5 ± 2.0
	W	98.4	0.13	1.93 ± 0.29	5.3 ± 0.4	0.8 ± 0.6	2.2 ± 1.6
Psychrophilic	CGLW	96.8	0.19	2.35 ± 0.21	5.6 ± 0.5	0.19 ± 0.21	0.22 ± 0.12
	CGLW + MA	94.9	0.34	1.4 ± 0.5	6.9 ± 1.2	0.17 ± 0.10	1.4 ± 1.1
	W	96.1	0.22	1.9 ± 1.2	5.3 ± 0.8	0.4 ± 1.1	0.01 ± 0.04
Yeast and mould	CGLW	97.1	0.16	2.8 ± 0.7	4.4 ± 1.1	0.26 ± 0.05	0.7 ± 0.7
	CGLW + MA	99.1	0.06	2.0 ± 0.5	5.6 ± 0.6	0.20 ± 0.03	0.5 ± 0.7
	W	99.3	0.03	3.42 ± 0.14	3.7 ± 1.0	0.30 ± 0.10	0.09 ± 0.14

* Values are means ± error standard
CGLW Dipped in aqueous solution of N-acetyl-L-cysteine at 1% (w/v), glutathione at 1% (w/v) and calcium lactate at 1% (w/v)
CGLW + MA Dipped in CGLW + malic acid at 2.5% (w/v)
W Dipped in sterile distilled water
k= Microorganisms initial count estimated by the model ($\log_{10}$ CFU/g)
A=*M*aximum microorganisms growth attained at the stationary phase ($\log_{10}$ CFU/g)
μ_{max} = Maximal growth rate [Δ log10 (CFU/g)/day]
λ= Lag time (days)
MSE= Mean Square Error

cysteine, glutathione and calcium lactate or by a combination of malic acid with those substances.

Shelf-life from a microbiological point view was calculated from the Gompertz equation modified by Zwietering et al. (1990) (Eq. 2) taking into account the Spanish regulation for hygienic processing, distribution and commerce of prepared meals (B.O.E, 2001), which indicates a maximum limit of mesophilic aerobic total count of 10^7 CFU/g at the expiry date. The microbiological shelf-life of those fresh-cut apples was also calculated, with regard to that limit for psychrophilic and yeast and mould populations. Since significant differences were not found between microbiological counts detected at both ripeness stages, the shelf-life was calculated from a mean of those counts (Table 4). Among the microbial populations, the mesophilic bacteria population was the more influential for the microbiological stability of fresh-cut apples. The maximum limit of mesophilic aerobic total count established by the Spanish regulation

(B.O.E, 2001) was reached at 10.1 days of storage in fresh-cut apples dipped in W (Table 4), whereas psychrophilic flora and yeasts and mould reached that level after 18.4 and 17.4 days respectively. A more prolonged shelf-life was reported by Vasantha- Rupasinghe et al. (2006), who found a total aerobic count equal to 4.61 and 5.01 Log CFU/g after 19 days of

Table 4. Shelf life from a microbiological point view of fresh-cut "Fuji" apple dipped in aqueous solutions of natural substances and stored at 5ºC predicted from the Gompertz equation.

Dipping condition	Population	Shelf life (days)
CGLW	Mesophilic	18.7
	Psychrophilic	20.6
	Yeast and Mould	21.9
CGLW +MA	Mesophilic	23.1
	Psychrophilic	27.1
	Yeast and Mould	25.0
W	Mesophilic	10.1
	Psychrophilic	18.4
	Yeast and Mould	17.4

CGLW Dipped in aqueous solution of N-acetyl-L-cysteine at 1% (w/v), glutathione at 1% (w/v) and calcium lactate at 1% (w/v)
CGLW + MA Dipped in CGLW + malic acid at 2.5% (w/v)
W Dipped in sterile distilled water

storage in fresh-cut "Empire" and "Crispin" apples respectively, starting with an initial population of 0.23 and 0.67 Log CFU/g. In the same way Corbo et al. (2004) indicated a shelf-life higher than 14 days for cut "Cactus" pear with an initial cell load equal to 4.10 Log CFU/g. In any of those cases the maximum value according to the Spanish regulation (10^7 CFU/g) was found. Fruit type, variety, initial microbial load in the fruit and practices of processing can be the cause of those differences found among the published work. On the other hand, microbiological stability strongly depended on the dipping treatment (Table 4). A prolonged microbiological stability (8.6 days) versus the cut-apple dipped in W was reached in cut-apple immersed in CGLW whereas a greater extension of that stability

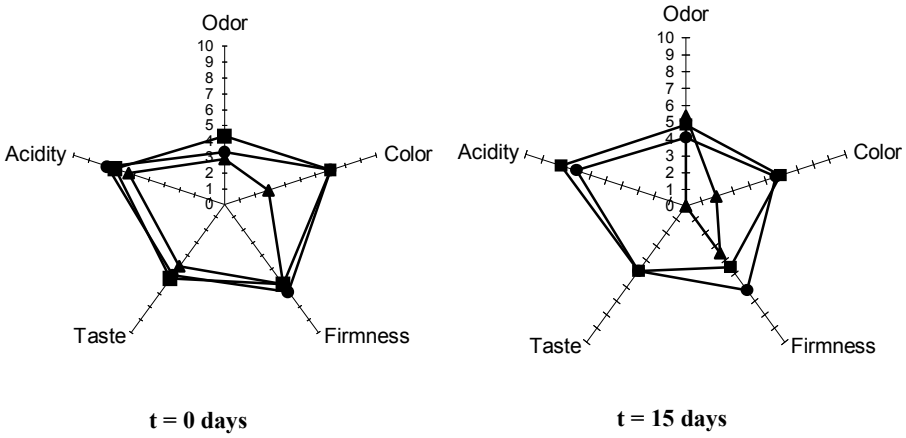
(13.0 days) was found using CGLW + MA. Therefore malic acid was the main responsible for the inhibitory activity of the dipping solutions.

The quality of the fresh-cut apple was significantly affected by parameters such as ethylene (Fig. 3) and ethanol production (Fig. 4), color (Fig. 5 and 6) and firmness (Table 2) , which limited its acceptability to 14 days in both fresh-cut partially ripe and ripe apple. However, the microbiological stability was mainly affected by dipping condition (Table 4), reaching the biggest extension when solutions of natural substances as CGLW (18.7 days) and CGLW + MA (23.1 days) were used.

Sensory evaluation of fresh-cut apples

Since partially ripe fresh-cut apple generated less ethylene and ethanol concentration and kept a better firmness and color than ripe fresh-cut apple, the former was selected to carry out sensory analyses. Storage time only appeared to have a significant ($p<0.05$) effect over odor and color characteristics. Odor at 15 days of storage time was ranked as more appealing for the panelists than at 0 days, whereas color was better scored at 0 days. Fresh-cut apple tissues continue to respire, thus undergoing changes during storage including production of phenolic compounds, which influences the fruit flavor and its acceptability by the consumer. On the other hand, minimal processing induces damage in the fruit tissue that allows the expsoure of some enzymes to oxygen, causing color changes and favoring browning. These facts explain why the panelists considered that fresh-cut apples had better odor at 15 days than 0 days but had better color at 0 days than 15 day.

Firmness, taste and acidity did not appear to be perceived differently by panelists (Fig. 8). Firmness variability during storage is mainly a consequence of the apple variety. On the other hand, taste and acidity depended directly on the apple variety and the used natural substances, but did not change during storage time. However color, firmness, taste and acidity were significantly different among dipping conditions (Fig. 8), the apple pieces treated with CGLW and CGLW + MA being better rated than the pieces treated with W. Stabilizing substances and malic acid highlight the taste of the fresh-cut apples.



t = 0 days **t = 15 days**

Figure 8.- Influence of the storage time on sensory characteristic of fresh-cut partially ripe "Fuji" apple dipped in aqueous solutions of N-Acetyl-L-cysteine at 1% (w/v), glutathione at 1% (w/v) and calcium lactate at 1% (w/v) (CGLW) (—●—), N-Acetyl-L-cysteine at 1% (w/v), glutathione at 1% (w/v), calcium lactate at 1% (w/v) and DL-malic acid 2.5% (w/v) (CGLW + MA) (—■—) and distilled water as control (W) (—▲—) packaged in air and stored at 5ºC during 30 days. The values are mean of 30 determinations.

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Inactivation of L. monocytogenes, S. Enteritidis and E. coli O157:H7 and shelf-life extension of fresh-cut pears using malic acid and quality stabilizing compounds

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ABSTRACT

The inactivation of *L. monocytogenes*, *S.* Enteritidis and *E. coli* O157:H7 inoculated in fresh-cut pears as well as the shelf-life extension of the product using malic acid and quality stabilizing compounds were investigated. Pear pieces were dipped in solutions containing N-acetyl-L-cysteine, glutathione and calcium lactate with and without D-L -malic acid and then inoculated or not with *L. monocytogenes*, *S.* Enteritidis or *E. coli* O157:H7 and stored at 5ºC for 30 days. Counts of pathogens and native flora were made during 30 days. Changes in headspace gas, firmness and color of the fresh-cut pears were also determined. Important reductions of *L. monocytogenes* (6.57 Log_{10}CFU/g), *S. Enteritidis* (6.60 Log_{10}CFU/g) and *E. coli* O157:H7 (2.62 Log_{10}CFU/g) at t = 0 days were achieved when malic acid was included into dipping solution. In addition, a shelf-life extension of fresh-cut pears was accomplished in those dipping conditions. In conclusion, the use combined of malic acid with quality stabilizing compounds can be a good alternative for assuring the safety and quality of fresh-cut pears.

Practical applications. The use of natural substances generally recognized as safe (GRAS) such as malic acid and N-acetyl-L-cysteine, glutathione and calcium lactate as antimicrobials and quality stabilizing compounds, respectively, can result suitable to fresh-cut products industry, since they can assure the safety and quality of those products, while improving their sensory attributes and maintaining, the fresh-like and healthy properties of these products greatly demanded by the consumers

Keywords: Fresh-cut pears; *L. monocytogenes*; *S.* Enteritidis; *E. coli* O157:H7; shelf-life; malic acid; N-acetyl-L-cysteine; glutathione; calcium lactate

INTRODUCTION

In the last years, consumption of ready-to-eat fresh fruits have rapidly increased (Corbo *et al.* 2000), presumably due, in part, to their characteristics of freshness, low caloric contents, commodity to be used and an active promotion of fruits and vegetables as basic components of a healthy diet. Nevertheless, it is well known that minimally processed fruit and vegetables are generally more perishable than the original raw materials. Injury stresses due to operations such as peeling, cutting, shredding, slicing, etc. lightly increase tissue respiration and lead to various biochemical deteriorations such as browning, off-flavor development and texture breakdown (Varoquaux and Wiley, 1994; Martín-Belloso *et al.* 2006) which decrease the fresh-cut fruit quality. Moreover, minimal processing may increase the microbial spoilage of the product (Pittia *et al.* 1999) as well as the consumer risk to acquire diseases, as a consequence of the pathogenic flora transferring from skin to the fruit tissue. In fact, the numbers of outbreak and cases by consumption of fresh-cut fruits and derivatives have increased in the last years. Eswaranandam *et al.* (2004) indicated that apple snack trays, assorted processed fruits and vegetables, and cut cantaloupe melon were recalled for possible contamination with *L. monocytogenes.* Likewise, Fan *et al.* (2005) reported one recall associated with fresh-cut apples due to a possible contamination with that microorganism. *E. coli* O157:H7 and *Salmonella* have been responsible of outbreaks associated with pear and melon fresh fruit, as well as apple juice and cider in recent years (Besser *et al.* 1993; Powell and Luedtke, 2000; USFDA, 2001; Harris *et al.* 2003; CDC, 2007).

Refrigerated storage as well as modified atmospheres (oxygen reduced) have been commonly used to extend the shelf-life of fresh- cut fruits. However, growth of some pathogenic bacteria such as *Listeria monocytogenes* and *E. coli* O157:H7 in fresh-sliced pears stored in both air and modified atmosphere at 4ºC have been reported (Corbo *et al.* 2005). Likewise, survival and/or growth of *E. coli* O157:H7, *Salmonella* serovars and *L. monocytogenes* in fresh-cut apples stored at different temperatures have been also reported (Fisher and Golden, 1998; Liao and Sapers, 2000; Lanciotti *et al.* 2003)

Numerous preservation strategies to avoid quality loss of fresh-cut fruits such as the use of additives (Raju and Bawa, 2006) added direct or indirectly to the fruits to reduce enzymatic browning (Ahvenainen, 1996; Laurila *et al.* 1998; Sapers and Miller, 1998), softening (Luna-Guzmán and Barrett, 2000; Gorny *et al.* 2002; Johnston *et al.* 2002), microbiological

spoil (Brul and Coote, 1999; Pittia *et al.* 1999; Corbo *et al.* 2004) and improving of the safety (Lanciotti *et al.* 2003; Derrickson-Tharrington *et al.* 2005) have been proposed in recent years. However effective strategies to avoid enzymatic browning, tissue softening, microbiological spoilage and reduced health risk in fresh-cut fruit is still a challenge for fresh-cut produce industry.

The objectives of this study were to evaluate the effectiveness of a combination of malic acid, N-acetyl-L-cysteine, glutathione and calcium lactate applied through dipping treatments to inactivate *L. monocytogenes*, *S.* Enteritidis and *E. coli* O157:H7 populations inoculated in fresh-cut pears as well as to evaluate its influences on the shelf-life of the product.

MATERIALS AND METHODS

Pears

Winter pears (*Pyrus communis* L.) cv. *Flor de Invierno* partially ripe (commercial ripeness) were provided by ACTEL, Lleida, Spain. A flesh characterization was carried out following the official methods of fruit juices and other vegetables and derivatives (B.O.E., 1988). Total acidity expressed as grams of citric acid/100 ml (0.35 ± 0.02), pH (Crison 2001 pH-meter; Crison Instruments S. A., Barcelona, Spain) (4.31 ± 0.04) and soluble solids content expressed as % (Atago RX-1000 refractometer; Atago Company Ltd, Japan) (14.3 ± 0. 21) were the evaluated parameters.

Dipping treatments

Two aqueous solutions containing: N-acetyl-L-cysteine (Acros Organics, New Jersey, USA) at 1% w/v, reduced glutathione (Acros Organics) at 1% w/v, calcium lactate pent-hydrate (Scharlau Chemie S.A., Barcelona, Spain) at 1% w/v into sterile distilled water (CGLW), and N-acetyl-L-cysteine at 1% w/v, reduced glutathione at 1% w/v, calcium lactate pent-hydrate at 1% w/v, D-L -malic acid (Scharlau Chemie S.A.) at 2,5% w/v into sterile distilled water (CGLW+MA) were prepared to avoid physicochemical and microbiological spoilage of fresh-cut pears. In addition, sterile distilled water (W) was used as control treatment. N-acetyl-L-cysteine, glutathione (Rojas-Graü *et al.* 2006a; Oms-Oliu *et al.* 2006), calcium lactate (Gorny *et al.* 2002) and D-L-malic acid (Raybaudi-Massilia *et al.* 2007) concentrations were selected according to previous studies. The

temperature of dipping solutions containing or not stabilizing substances and/or malic acid was room temperature (18ºC).

Strains and inoculums preparation

Listeria monocytogenes 1.131 (CECT 932) and *Escherichia coli* O157:H7 (CECT 4267) from the Spanish Type Culture Collection (University of Valencia, Valencia, Spain), and *Salmonella* Enteritidis 1.82 (NCTC 9001) from the National Collection of Type Culture (Central Public Health Laboratory; London, UK) were maintained in tryptone soy agar (TSA) (Biokar Diagnostics. Beauvais, France) slants at 5ºC until its use. Stock cultures of *L. monocytogenes* and *E. coli* O157:H7 were grown in tryptone soy broth (TSB) (Biokar Diagnostics) with 0.6% yeast extract (Biokar Diagnostics); whereas, *S.* Enteritidis was cultured in TSB. *E. coli* O157:H7 and *S.* Enteritidis were incubated at 37ºC with continuous agitation for 11 hours at 120 rpm, while *L. monocytogenes* was incubated at 35ºC with continuous shaking for 15 hours at 200 rpm to obtain cells in early stationary growth phase. The maximum growth for *L. monocytogenes*, *S.* Enteritidis and *E. coli* O157:H7 was 10^9, colony forming units/milliliter (CFU/ml). Concentrations were then adjusted to 10^8 CFU/ml using saline peptone water (Biokar Diagnostics) at 0.1% plus sodium chloride (Scharlau Chemie, S.A.) at 0.85%.

Pears processing and packaging

For challenge studies, pears maintained at room temperature (18ºC) for 12 hours were sanitized by immersion in an aqueous solution of chlorine (300 µl/l, pH 6.8); then washed with potable water and finally dried with absorbent paper. Clean fruits were cut in the poles with a knife and then with a cylindrical hollow instrument of stainless steel of sharpened brink in cylindrical pieces of 1.40 cm diameter x 2.00 cm height taken out from the fruit mesocarp (approximately 5 cylinders per pear). The cut pears were divided in three parts and next dipped for 2 min in each dipping solution (CGLW, CGLW+MA, W) with a ratio fruit: solution equal to 1:2 and constant agitation using a magnetic stirrer. After natural drained by 2 minutes, ten cylinders of pear with a total weight of 50 g were placed into polypropylene trays of 173 x 129 x 35 mm (Ilpra Systems España, S.L, Barcelona, Spain). Afterward, the pear pieces were inoculated uniformly by spreading 500 µl of *L. monocytogenes, S* Enteritidis or *E. coli* O157:H7 stock cultures (10^8 CFU/ml) over its entire upper surface with a sterile

micropipette. The trays were then wrap-sealed with a 64 μm thick polypropylene film with a water vapor permeability of 142.86 fmol $s^{-1}m^{-2}$ kPa^{-1} at 38ºC and 90% R.H., O_2 permeability of 52.38 fmol $s^{-1}m^{-2}$ kPa^{-1} at 23ºC and 0% R.H., and CO_2 permeability of 2.38 fmol s^{-1} m^{-2} kPa^{-1} at 23ºC and 0% R.H. (Tecnopack SRL, Mortara, Italy) using a vacuum compensated packaging machine (ILPRA Food Pack Basic V/6, Ilpra S. CP. Vigevono, Italy). Trays were filled with air, sealed, and stored in refrigeration at 5ºC for 30 days. On the other hand, 50 g of non-inoculated fresh-cut pears processed in the same way as previously indicated were packed, sealed and stored at 5ºC for 30 days for shelf-life and sensory studies.

Trays of fresh-cut pears dipped in each solution (CGLW, CGLW+MA, W) for microbiological challenge studies (20 trays for *L. monocytogenes,* 20 trays for *S. Enteritidis* and 20 trays for *E. coli* O157:H7 for each dipping condition), shelf-life estimation (10 trays for physicochemical determinations and 10 trays for microbiological analysis for each dipping condition) were prepared to be analyzed at 0, 7, 14, 21 and 30 days of refrigerated storage. In addition, 12 trays (6 for 0 day and 6 for 15 days of storage for each dipping condition) were prepared to evaluate the sensory characteristics.

***L. monocytogenes*, *S.* Enteritidis and *E. coli* O157:H7 survival**

Fifty grams of inoculated fresh-cut pears were diluted with 450 ml of buffered peptone water (pH 7.2) (Biokar Diagnostics) and homogenized in a masticator (IUL Instruments, Barcelona, Spain) for 1 min. Serial dilutions were prepared and spread at reason of 0.1 ml on Palcam, Hektoen and MacConkey-Sorbitol agar plates in duplicate for *L. monocytogenes*, *S.* Enteritidis and *E. coli* O157:H7 counts, respectively. The plates were incubated for 24-48 h at 35-37ºC. Culture media were provided by Biokar Diagnostics.

***L. monocytogenes*, *S.* Enteritidis and *E. coli* O157:H7 recovery.**

Injured cells of *L. monocytogenes*, *S.* Enteritidis and *E. coli* O157:H7 from fresh-cut pears treated with a dipping solution containing malic acid and quality stabilizing substances were recovered using buffered peptone water (pH 7.2) and maintaining for 20 min at 35-37ºC. Subsequently, an aliquot of 0.1 ml was spread plated on Palcam, Hektoen and MacConkey-Sorbitol agars for *L. monocytogenes*, *S.* Enteritidis and *E.*

coli O157:H7 counts, respectively. Those plates were then incubated for 24-48h at 35-37ºC. The recovery medium was selected according to Liao and Fett (2005), whereas recovery time (20 min) was selected taking into account the generation time of each microorganism from growth curves previously made in the laboratory (not shown data); where repairing of injured cell without cellular multiplication was assumed.

Mesophilic, psychrophilic and yeast and moulds counts

Mesophilic and psychrophilic microorganisms counts in non-inoculated fresh-cut pears were made according to the ISO 4833:1991 (1991) guideline using plate count agar (PCA) (Biokar Diagnostics) and pour plate method. The plates of psychrophilic microorganisms were incubated at 5ºC for 10-14 days, whereas mesophilic microorganisms were incubated at 35ºC for 48 h. On the other hand, yeasts and molds counts were made according to the ISO 7954:1987 (1987) guideline using chloramphenicol glucose agar (CGA) (Biokar Diagnostics) and spread plate method. The plates were incubated at room temperature (18ºC) for 3-5 days. All the microbial counts were expressed as $\log_{10}$ CFU/g and the reported values are the mean of four determinations ± standard deviation.

Headspace gases analysis

The gas composition of the package headspace was determined using a Micro-GP CP 2002 gas analyzer (Chrompack International, Middelburg, Netherlands) equipped with a thermal conductivity detector. A sample of 1.7 ml was automatically withdrawn from the headspace atmosphere. Portions of 0.25 and 0.33 µl were injected for O_2 and CO_2 determination, respectively. The O_2 content was analyzed with a CP-Molsieve 5Å packed column (Chrompack International, Middelburg, Netherlands) (4m x 0.32mm, df =10mm) at 60ºC and 100 kPa. On the other hand, a Pora-PLOT Q column (Chrompack International, Middelburg, Netherlands) (10m x 0.32mm, df =10mm) was held at 70ºC and 200 kPa for CO_2, ethylene, and ethanol quantification. A pair of trays randomly taken was analyzed in duplicated for each dipping condition at 0, 7, 14, 21 and 30 days. Thus, the reported values are the mean of four determinations ± standard deviation.

Color determination

The color of the fresh-cut pears was determined in a pair of trays for each dipping condition randomly chosen using a tri-stimulus Minolta CR-400 Chroma Meter (Konica Minolta Sensing, INC. Osaka, Japan) using the illuminant D75 and observation angle of 10º, calibrated with a standard white plate (Y = 94.00, x = 0.3158, y = 0.3322). Three readings of L* (Lightness), a* (green chromaticity) and b* (yellow chromaticity) coordinates were recorded for each pear cylinder. A total of 10 cylinders of fresh-cut pears were analyzed by pair of tray. Therefore, the reported values are the mean of thirty determinations ± standard deviation. Numerical values of a* and b* parameters were employed to calculated hue angle (hº) through the equation 1:

$$h^o = \arctan \frac{b^*}{a^*} \quad (1)$$

Firmness determination

The firmness determination was performed in ten cylinders coming from a pair of fresh-cut pears trays for each dipping condition using a TA-TX2 Texture Analyzer (Stable Micro Systems LTD. Surrey, England) at the following conditions: Pre-test speed: 2 mm/s, test speed: 5.0 mm/s, post-test speed: 5.0 mm/s and penetration distance: 10 mm. The resistance of penetration was measured as the necessary strength for a cylindrical probe of 4 mm of diameter and plane basis to penetrate a cylindrical sample of pear flesh of 2.00 cm height. Five pieces of each tray were analyzed. The reported values are the mean of 10 measurements ± standard deviation.

pH determination

The pH of fresh-cut pears immersed in different dipping solutions was determined weekly using a pH-meter (Crison Instruments S. A. Barcelona, Spain) for evaluating the influence of the added substances over this parameter as well as possible changes through the storage time. The reported values are the mean of values obtained in 5 determinations (each one in duplicated) made throughout storage time (n= 10) ± SD for each dipping condition (Table 1).

TABLE 1
pH VALUES OF FRESH-CUT PEARS INOCULATED OR NOT WITH *L. MONOCYTOGENES, S.* ENTERITIDIS AND *E. COLI* O157:H7 AND DIPPED IN AQUEOUS SOLUTIONS OF QUALITY STABILIZING COMPOUNDS WITH AND WITHOUT MALIC ACID

Dipping condition	pH [a]		
	Non-inoculated	Inoculated with *L.monocytogenes*	Inoculated with S. Enteritidis
CGLW [b]	4.84 ± 0.07	4.75 ± 0.09	4.56 ± 0.14
CGLW + MA [c]	4.4 ± 0.4	4.0 ± 0.4	4.2 ± 0.4
W [d]	4.75 ± 0.14	4.65 ± 0.02	4.63 ± 0.02

[a] Means of values obtained in 5 determinations in duplicate made throughout storage time (n= 10) ± SD
[b] CGLW Dipped in aqueous solution of N-acetyl-L-cysteine at 1% (w/v), glutathione at 1% (w/v) and calcium lactate at 1% (w/v)
[c] CGLW + MA Dipped in CGLW + malic acid at 2.5% (w/v)
[d] W Dipped in sterile distilled water

Sensory evaluation

Fresh-cut pears dipped in CGLW, CGLW+MA and W were used to carry out sensory analyses in a similar way as Raybaudi-Massilia *et al.* (2007). Samples of fresh-cut pears from dipping treatments were prepared to be evaluated the same day of the sample preparation (t = 0) and after 15 days of storage at 5ºC. Thirty volunteers aged between 20-50 years old, who like and eat frequently pear did make the sensory tests. A total of 6 samples (one sample per dipping condition (CGLW, CGLW + MA and W) and per storage time (t = 0 and t = 15 days) consisted of 2 cylinders of fresh-cut pears each one, labeled with 3 numbers were randomly provided to the panelists, who scored acceptability of odor, color, taste, acidity and firmness characteristics on a structured 10 cm hedonic scale labeled from "extremely unpleasant" (0) to "extremely pleasant" (10). A glass containing potable water and pieces of non-salted cracker were provided to panellists for eliminating the residual taste between samples.

Predictive modeling and statistical analysis

Mesophilic, psychrophilic and yeasts and moulds growth in non-inoculated fresh-cut pears, expressed as $\log_{10}$ CFU/g were modeled according to the Gompertz equation modified by Zwietering *et al.* (1990)

(Equation 2), whereas microbiological shelf-life (***SL***) was calculated through the Equation 3 obtained from modified Gompertz model (Equation 2). The latter equation consider as maximum limit of mesophilic aerobic total count at expiry date the value allowed by the Spanish regulation for hygienic processing, distribution and commerce of prepared meals (B.O.E, 2001) which is 10^7 CFU/g. This limit was also considered for psychrophilic and yeast and mold populations.

$$Y = k + A \cdot exp\left\{-exp\left[\left(\mu_{max} \cdot 2.7182 / A\right)\left(\lambda - t\right) + 1\right]\right\} \quad (2)$$

$$SL = \lambda - \frac{A\left\{ln\left[-ln\left(\frac{log(10^7) - k}{A}\right)\right] - 1\right\}}{\mu_{max} \cdot 2.7182} \quad (3)$$

where Y is the count of microorganisms ($\log_{10}$ CFU/g) for a given time, k is the microorganism initial count estimated by the model ($\log_{10}$ CFU/g), A is the maximum microorganism growth attained at the stationary phase ($\log_{10}$ CFU/g), μ_{max} is the maximal growth rate [$\Delta \log_{10}$ (CFU/g)/day], λ is the lag time (days), t is the storage time (days) and SL is shelf-life time.

In addition, a multifactor ANOVA with posterior Multiple Range Test was used to find significant differences ($p < 0.05$) among storage time and dipping condition on microbiological counts, headspace gas, firmness, color and sensory evaluation profile.

RESULTS AND DISCUSSION

Effect of dipping condition and storage time on pathogenic bacteria inoculated in fresh-cut pears.

L. monocytogenes, S. Enteritidis and *E. coli* O157:H7 counts were significantly ($p < 0.05$) affected by dipping conditions at the sample preparation day (t = 0 days). Fresh-cut pears dipped in CGLW and W and then inoculated with *L. monocytogenes*, *S.* Enteritidis or *E. coli* O157:H7 did not show significant changes ($p > 0.05$) in bacterial counts after processing (t = 0 days), whereas, those fresh-cut pears dipped in CGLW+MA exhibited significant decreases ($p < 0.05$) in those populations (Fig. 1). Those results are in according to those reported by DiPersio *et al.* (2003) who indicated that populations of *Salmonella* inoculated in apple slices were not significantly ($p > 0.05$) reduced by immersion in W. However, those results differ from those reported by Derrickson-Tharrington *et al.* (2005) who indicated that a reduction about 0.9-1.0 Log CFU/g in fresh sliced apples immersed in water for 10 min was observed. This reduction is due probability to the processing and inoculation method used by those authors, since fresh-sliced apples were first inoculated with *E. coli* O157:H7 and then immersed in water, suggesting that microbial reductions could be caused by the rinse of fresh-cut fruit in water. In addition, a higher reduction of *E. coli* O157:H7 (2.62 Log_{10} CFU/g) in fresh-cut pears at t = 0 days using a dipping solution containing malic acid and stabilizing substances was found in this study in comparison with those reported by Derrickson-Tharrington *et al.* (2005) (0.9-1.3 Log_{10} CFU/g) using acidic dipping solutions containing 2.8% ascorbic acid, 1.7% citric acid or 50% commercial lemon juice with or without preservatives (0.9-1.3 Log_{10} CFU/g). Those differences may be consequence of the kind of acid and concentration used.

Characteristic colonies of *L. monocytogenes* and *S.* Enteritidis were not recovered from fresh-cut pears samples treated with a CGLW+MA solution after a recovery step in buffered peptone water for 20 min at 35-37ºC, thus confirming the immediate bactericidal effect of the malic acid over those microorganisms. Therefore, more than 5 log reductions of those pathogens could be reached from the sample preparation day (t = 0 day). Nevertheless, a higher acid-resistance of *E. coli* O157:H7 population to malic acid was observed; since, a survival fraction of 3.84 Log_{10} CFU/g of this microorganisms in fresh-cut pears immersed in CGLW + MA after processing (t = 0 days) was detected (Fig. 1c). Similar acid-resistance of *E. coli* O157:H7 have been reported by others researchers (Benjamin and

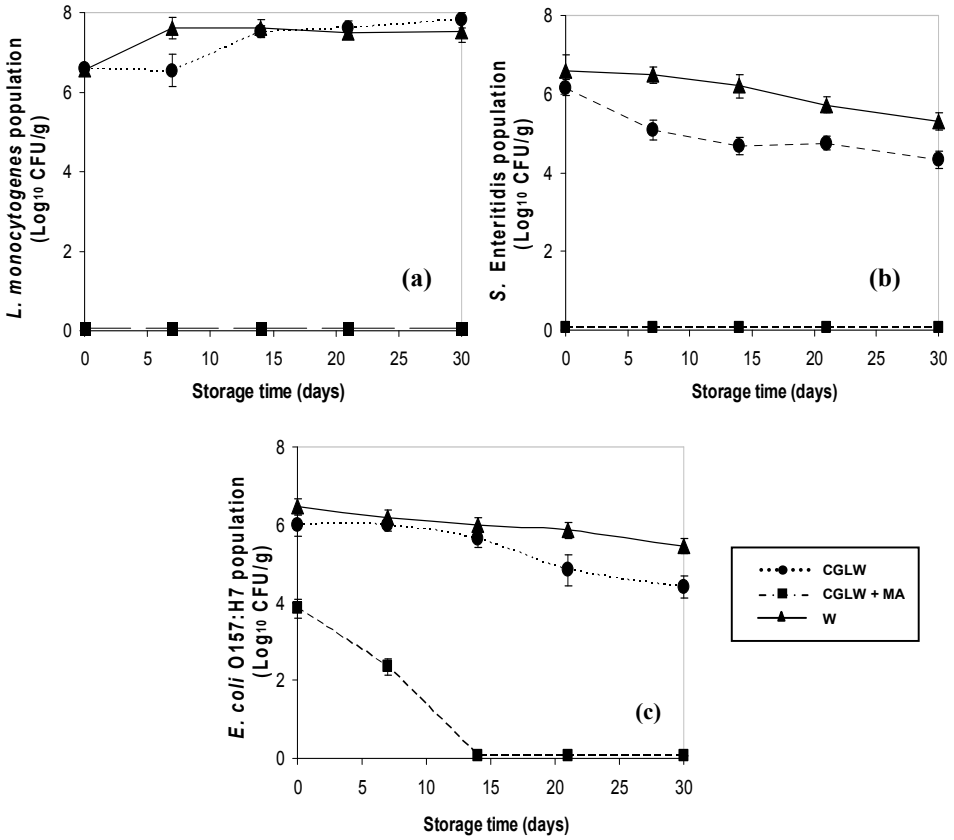

Figure 1.- Behavior of *L. monocytogenes* (a), *S.* Enteritidis (b) and *E. coli* O157:H7 (c) in fresh-cut pears inoculated after dipped in aqueous solutions of n-acetyl-l-cysteine at 1% (w/v), glutathione at 1% (w/v) and calcium lactate at 1% (w/v) (CGLW), n-acetyl-l-cysteine at 1% (w/v), glutathione at 1% (w/v), calcium lactate at 1% (w/v) and dl-malic acid 2.5% (w/v) (CGLW + MA), and distilled water (W) packed in air and stored at 5ºc during 30 days. detection limit: 2 $\log_{10}$ cfu/g. values are mean of 4 determinations ± SD. *L. monocytogenes* and *S.* Enteritidis were not detected in fresh-cut pears dipped in CGLW + ma from the sample preparation day (t = 0 days) after a recovering step, whereas *E. coli* O157:H7 was undetectable from t = 14 days after that step.

Data, 1995; Derrickson-Tharrington *et al.* 2005; Ingham *et al.* 2006). In such sense, Lin *et al*. (1996) indicated that three systems are involved in the acid tolerance of *E. coli* O157:H7, including an acid-induced oxidative system, an acid-induced arginine-dependent system, and a glutamate-dependent system.

Malic acid is an organic acid of low lipid solubility (Leo *et al.* 1971) and consequently its entrance to the cell could be limited, since the cell membrane is little permeable to polar compounds (Lücke, 2003). Therefore, a decrease in the medium pH could explain its antimicrobial effect as was suggested by Beuchat and Golden (1989). However, some authors have found that effectiveness of the organic acids can vary depending on its molecular weight. Eswaranandam *et al.* (2004) indicated that undissociated smaller molecules of malic (134.09 Dalton) and lactic (90.08 Dalton) acids may entry into the bacterial cells easily and change the internal pH of the microorganism, thus showing, higher antimicrobial activity than undissociated larger molecules of citric (192.13 Dalton) and tartaric (150.09 Dalton) acids, which may not entry toward the cell inside effectively.

According to our results, a simple reduction of medium pH could explain the bactericidal effect of malic acid over pathogenic populations, since pH values in fresh-cut pears treated with CGLW + MA were significantly ($p < 0.05$) lower than in those fresh-cut pears dipped in CGLW or W (Table 1). However, a diminution of the intracellular pH due to the diffusion of the smaller malic acid molecules into the cell also could explain to inactivation of those microorganisms. Lou and Yousef (1999) indicated that the antimicrobial action of organic acids is attributed to cytoplasm acidification, as well as the specific antimicrobial effect of the particular anionic species. Those authors also indicated that undissociated organic acids can pass through the cell membrane and dissociate inside the cytoplasm, and interfere with metabolic processes of the microbial cell.

Populations of *L. monocytogenes, S.* Enteritidis and *E. coli* O157:H7 inoculated in fresh-cut pears showed different behavior through the storage time. Thus, a slight growth of *L. monocytogenes* on fresh-cut pears dipped in CGLW or W was observed during storage, showing an increase of its population from 6.57 Log_{10} CFU/g (t = 0 days) to 7.81 or 7.60 Log_{10} CFU/g, respectively after 7 days of storage at 5ºC. A similar increase of *L. monocytogenes* in fresh-sliced "Cactus" pear packed under air (1.20 Log_{10} CFU/g) or passive-modified atmosphere (65% N_2, 30% CO_2, 5% O_2) (1.50 Log_{10} CFU/g) over 14 days of storage at 4ºC was reported by Corbo *et al.* (2005). On the other hand, fresh-cut pears dipped in

CGLW+MA reduced the population of *L. monocytogenes* to undetectable levels and its growth throughout storage time was not observed. The growth of the *L. monocytogenes* in fresh-cut pears dipped in CGLW and W demonstrated that this microorganism can survive and grow for at least 30 days of storage in this kind of products assisted by its psychrotrophic nature (Lou and Yousef, 1999).

In contrast, growth of *S.* Enteritidis and *E. coli* O157:H7 populations in fresh-cut pears stored at 5ºC through the storage time were not detected irrespectively of the used dipping solution (Fig. 1b and 1c). These results differed from those reported by Corbo *et al.* (2005) who indicated a proliferation of *E. coli* O157:H7 in fresh-sliced cactus-pears stored at 4 and 8ºC. The differences found could be due to variations among strains and variety of fruit used. On the other hand, significant reductions ($p < 0.05$) of *S.* Enteritidis and *E. coli* O157:H7 populations occurred through the 30 days of storage, being the greatest observed survival in those fresh-cut pears dipped in W, followed by those immersed in CGLW, whereas, in CGLW+MA populations of *S.* Enteritidis and *E. coli* O157:H7 were undetectable from 0 and 14 days, respectively (Fig. 1b and 1c). Characteristic colonies of *E. coli* O157:H7 in fresh-cut pears dipped in CGLW+MA were not found after a recovering step at $t \geq 14$ days of storage, thus, more than 5 log reductions of this microorganism were achieved. Our results demonstrated that not only malic acid has a powerful bactericidal effect, but also the stabilizing substances (N-acetyl-L-cysteine, glutathione and calcium lactate) incorporated into the dipping solution showed some antimicrobial effect, since differences during storage time were found between the *S.* Enteritidis and *E. coli* O157:H7 counts in fresh-cut pears dipped in CGLW and W, being more significant for *S.* Enteritidis population than for *E. coli* O157:H7 (Fig. 1b and 1c).

The reduction in *S.* Enteritidis and *E. coli* O157:H7 counts observed throughout storage in fresh-cut pears dipped in W suggests that other factors such as storage temperature (5ºC), competition of the native microflora or headspace gas composition of the trays might influence the pathogen survival. In this way, Raybaudi-Massilia *et al.* (2007) reported significant changes ($p < 0.05$) in headspace gas composition of fresh-cut apples dipped in W without any substance added, indicating that an important decrease of O_2 and a significant increase of CO_2 was observed in fresh-cut apples during 30 days of storage at 5ºC. However, it is well known that *Salmonella* species and *E. coli* O157:H7 are facultative anaerobic microorganisms, and thus, headspace gas composition could not be the main cause of the reduction of their populations. Likewise, these authors

indicated that a meaningful growth of mesophilic, psychrophilic and yeasts and molds populations occurred in fresh-cut apples during the storage. Therefore, we consider that storage temperature and competition of the native microflora might be the main factors that caused the reduction on *Salmonella* and *E. coli* O157:H7 counts in fresh-cut pears dipped in W through storage. Similarly, Liao and Sapers (2000) reported a slight reduction of *S.* Chester on non-treated apple disks stored at 8ºC after 3 days of storage.

Microbial stability of non-inoculated fresh-cut pears

The growth of mesophilic and psychrophilic microorganisms as well as yeasts and molds in non-inoculated fresh-cut pears dipped in stabilizing solutions with or without malic acid or water and stored at 5ºC for 30 days was successfully modelled with the Gompertz equation as modified by Zwietering et al. (1990) (Eq. 2). The behavior of microorganism populations under different treatment conditions is shown in Fig. 2, and parameters defined by the model such as initial load (K), maximum growth attained at the stationary phase (A), maximal growth rate (μ_{max}) and lag phase (λ) are provided in Table 2.

A significant increase ($p < 0.05$) of native flora in fresh-cut pears was observed throughout the storage time irrespectively of the dipping condition used (Fig. 2). Similar behavior have been reported in fresh-cut pears by others researchers (Soliva-Fortuny and Martín-Belloso, 2003; Corbo *et al.* 2004; Soliva-Fortuny *et al.* 2004). In addition, significant differences ($p < 0.05$) for each population (mesophilic, psychrophilic and yeasts and molds) were detected among dipping conditions, showing the fresh-cut pears dipped in CGLW + MA the lowest populations (Fig. 2) and the fresh-cut pears immersed in W the highest.

Mesophilic population ranged from 3.16 to 8.37 Log_{10} CFU/g in fresh-cut pears dipped in W, from 2.72 to 7.19 Log_{10} CFU/g in those cut-pears immersed in CGLW and from 1.97 to 5.40 Log_{10} CFU/g in those submerged in CGLW + MA (Fig. 2a) after 30 days of storage at 5ºC. The mesophilic flora detected in fresh-cut pears immersed in W appeared to be constituted by bacteria more than yeasts and molds, because similar counts of mesophilic (Fig. 2a) and psychrophilic (Fig. 2b) populations, and lower counts of yeasts and molds (Fig. 2c) were detected in cut-pears dipped in W. Jay *et al.* (2005) and Brackett (2001) reported that the fruits may be able to support the bacteria, yeasts and molds growth due to their nutrient content; however, the pH of the fruits is below the level that generally

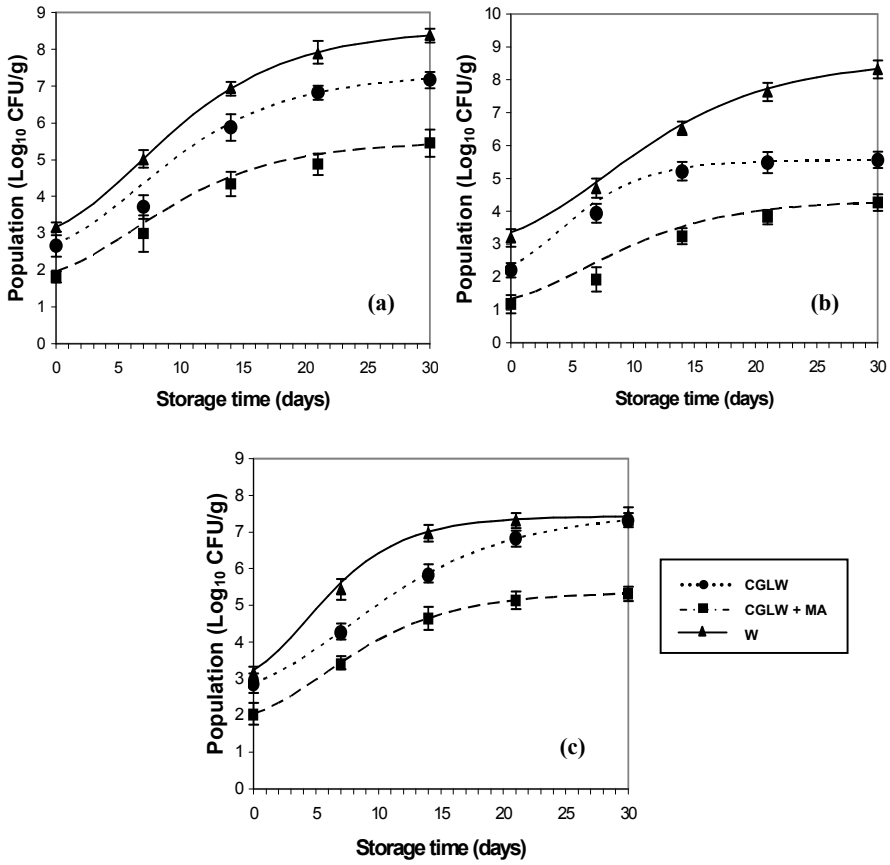

Figure 2.- Influence of the dipping condition and storage time on mesophilic bacteria (a), psychrophilic bacteria (b), yeasts and molds (c) growth (log_{10} cfu/g) in non-inoculated fresh-cut pears dipped in aqueous solutions of N-acetyl-L-cysteine at 1% (w/v), glutathione at 1% (w/v) and calcium lactate at 1% (w/v) (CGLW), N-acetyl-L-cysteine at 1% (w/v), glutathione at 1% (w/v), calcium lactate at 1% (w/v) and D-L-malic acid 2.5% (w/v) (CGLW + MA) and distilled water as control (W) packed in air and stored at 5ºc during 30 days. the values are mean of 4 determinations ± SD.

TABLE 2
GOMPERTZ PARAMETERS THAT DESCRIBE THE GROWTH OF MESOPHILIC, PSYCHROPHILIC, YEASTS AND MOLDS IN NON-INOCULATED FRESH-CUT PEARS DIPPED IN AQUEOUS SOLUTIONS OF QUALITY STABILIZING COMPOUNDS WITH AND WITHOUT MALIC ACID AND STORED AT 5ºC FOR 30 DAYS

Population	Dipping condition	R^2	MSE	Gompertz parameters [a]			
				k	A	μ_{max}	λ
Mesophilic	CGLW [b]	99.71	0.014	2.40±0.21	4.93±0.29	0.279±0.019	0.51±0.23
	CGLW + MA [c]	97.88	0.065	1.72±0.29	3.8±0.4	0.22±0.04	2.02±0.43
	W [d]	99.97	0,002	2.79±0.10	5.75±0.13	0.319±0.007	0.04±0.02
Psychrophilic	CGLW	99.72	0.008	2.13±0.12	3.44±0.15	0.32±0.03	1.3±0.9
	CGLW + MA	99.77	0.005	1.12±0.07	3.21±0.12	0.189±0.011	2.7±0.7
	W	99.96	0.002	2.99±0.06	5.63±0.13	0.273±0.006	0.7±0.5
Yeasts and molds	CGLW	99.59	0.019	2.55±0.27	5.0±0.4	0.248±0.019	0.20±0.07
	CGLW + MA	99.74	0.007	1.81±0.16	3.55±0.21	0.234±0.016	0.23±0.0.2
	W	99.88	0.005	2.95±0.16	4.48±0.19	0.392±0.026	0.54±0.08

[a] Values are means ± error standard
[b] CGLW Dipped in aqueous solution of N-acetyl-L-cysteine at 1% (w/v), glutathione at 1% (w/v) and calcium lactate at 1% (w/v)
[c] CGLW + MA Dipped in CGLW + malic acid at 2.5% (w/v)
[d] W Dipped in sterile distilled water
k= Initial bacterial counts estimated by the model ($\log_{10}$ CFU/g)
A=*M*aximum microorganisms growth attained at the stationary phase ($\log_{10}$ CFU/g)
μ_{max}= Maximal growth rate [Δ log10 (CFU/g)/day]
λ= Lag time (days)
R^2 = Correlation coefficient
MSE= Mean Square Error

favors bacterial growth, explaining the general absence of bacteria on the incipient spoilage of the majority of fruits, with the exception of pears, which some time can undergo spoilage by bacteria as *Erwinia* spp. Contrary to the observed in fresh-cut pears dipped in W, mesophilic population in fresh-cut pears dipped in CGLW or CGLW + MA appeared to be constituted in its majority by yeasts and molds; since, comparable counts were found between those populations (Fig. 2). Antibacterial effect of malic acid and stabilizing compounds and reduction in pH of fresh-cut apples in solution containing malic acid could explain differences found among the predominant flora in fresh-cut pears immersed in W and dipping solutions of CGLW and CGLW + MA which inhibits the bacterial growth more than yeasts and molds (Fig.2). On the other hand, the psychrophilic and yeasts and molds growth was more affected in those fresh-cut pears dipped in CGLW and CGLW + MA than in those dipped in W (Fig. 2).

The modified Gompertz model was adequately fitted the experimental data, since the coefficients of correlation ranged from 97.88 to 99.97 % as is shown in Table 2..Quality stabilizing added into dipping solutions delayed the proliferation of the mesophilic, psychrophilic and yeast and molds populations, since lower A and μ_{max} values as well as longer λ were observed, being even more affected those parameters with the addition of malic acid into the dipping solution (Table 2).

These results demonstrated that the substances (N-acetyl-L-cysteine, glutathione and calcium lactate) used for avoiding the loss of quality of the fresh-cut pears from a physicochemical point of view had an additional antimicrobial effect. However that antimicrobial effect was smaller than the effect caused by the addition of malic acid to the dipping solution. Jay et al. (2005) reported that, although the antioxidant agents are used in foods primarily to prevent the auto-oxidation of lipids, some of them, as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), ethylenediaminetetraacetic acid (EDTA), sodium citrate, lauric acid, monolaurin, etc, have demonstrated to possess antimicrobial activity against a wide range of microorganisms.

Modified Gompertz equation was also used to calculate the shelf-life from a microbiological point of view of fresh-cut pears immersed in different dipped solutions (Eq. 3) taking into account the Spanish regulation for hygienic processing, distribution and commerce of prepared meals (B.O.E., 2001), which indicates a maximum limit of mesophilic aerobic total count of 10^7 CFU/g at expiration date. The shelf-life of fresh-cut pears immersed in W (control) was mainly limited by the mesophilic population, since this population achieved 10^7 CFU/g at 8.92 days of refrigerated storage, whereas psychrophilic and yeasts and molds populations reached that levels at 10.26 and 11.15 days, respectively (Table 3). That fact corroborates that the spoilage of fresh-cut pears mainly occurred by bacterial action as reported by Jay et al. (2005) and Brackett (2001), who indicated that pears are an exception among the fruit, which some time undergo bacterial more than mold spoilage. A longer shelf-life was reported by Corbo *et al*. (2004) who indicated that cut "Cactus" pear had a shelf life over 14 days.

In contrast, shelf-life of fresh-cut pears dipped in CGLW was mainly limited by yeasts and molds population (17.52 days) rather than mesophilic (19.31 days) and psychrophilic (> 30 days) populations (Table 3). Unlike fresh-cut pears immersed in W and CGLW, fresh-cut pears dipped in CGLW + MA showed to maintain the microbial growth below the permitted maximum limit by more than 30 days. Those results

demonstrated that stabilizing and malic acid had a bactericidal effect which favored the yeast and molds growth in fresh-cut pears, possibly as a consequence of a lower competition among bacteria and yeasts and molds for the nutrients. Despite that, a more prolonged shelf-life was accomplished with the addition of stabilizing compounds and malic acid to the dipping solutions.

TABLE 3
SHELF-LIFE FROM A MICROBIOLOGICAL POINT OF VIEW OF FRESH-CUT PEARS DIPPED IN AQUEOUS SOLUTIONS OF QUALITY STABILIZING COMPOUNDS WITH AND WITHOUT MALIC ACID AND STORED AT 5ºC FOR 30 DAYS.

Dipping condition	Population	Shelf life[a] (days)
CGLW[b]	Mesophilic	19.31
	Psychrophilic	> 30
	Yeasts and Molds	17.52
CGLW +MA[c]	Mesophilic	> 30
	Psychrophilic	> 30
	Yeasts and Molds	> 30
W[d]	Mesophilic	8.92
	Psychrophilic	10.26
	Yeasts and Molds	11.15

[a] Predicted from Gompertz equation Shelf-life
[b] CGLW Dipped in aqueous solution of N-acetyl-L-cysteine at 1% (w/v), glutathione at 1% (w/v) and calcium lactate at 1% (w/v)
[c] CGLW + MA Dipped in CGLW + malic acid at 2.5% (w/v)
[d] W Dipped in sterile distilled water
10^7 CFU/g was used as maximal limit for shelf-life calculation of all populations

Headspace gas changes

Decreases in the oxygen concentrations and increases in the carbon dioxide, ethanol and ethylene production in fresh-cut pears were observed. The changes were significantly affected ($p < 0.05$) by the dipping condition and storage time. A progressive decrease in the oxygen concentration of fresh-cut pears dipped in W from the initial level (t = 0 days) until the maximum storage time (t = 30 days) was observed (Fig. 3a), whereas in

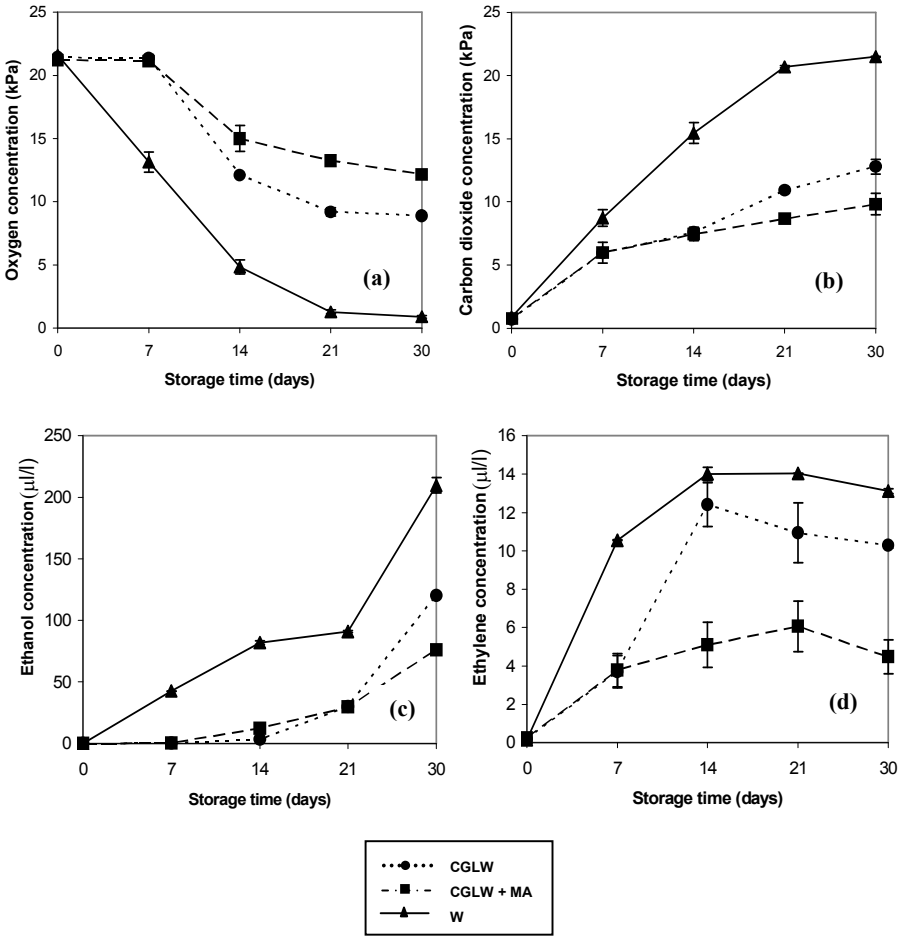

Figure 3.- Changes in oxygen (a), carbon dioxide (b), ethanol (c) and ethylene (d) concentration in headspace of trays with fresh-cut pears dipped in aqueous solutions of N-acetyl-L-cysteine at 1% (w/v), glutathione at 1% (w/v) and calcium lactate at 1% (w/v) (CGLW), N-acetyl-L-cysteine at 1% (w/v), glutathione at 1% (w/v), calcium lactate at 1% (w/v) and D-L-malic acid 2.5% (w/v) (CGLW + MA) and distilled water as control (W) packed in air and stored at 5ºc during 30 days. the values are mean of 4 determinations ± SD.

fresh-cut pears immersed in CGLW or CGLW + MA, the oxygen levels were maintained constant during the first seven days of storage and then progressively decreased throughout the time (Fig. 3a). On the other hand, higher oxygen consumption through the storage time was observed for fresh-cut pears immersed in W than in those fresh-cut pears dipped in CGLW + MA and CGLW (Fig. 3a). A higher carbon dioxide and ethanol production was also observed through the storage time in fresh-cut pears dipped in W than in those fresh-cut pears immersed in CGLW or CGLW + MA (Fig. 3b and 3c). That behavior in headspace gases have been also reported by Soliva-Fortuny *et al.* (2004) in fresh-cut "Conference" pears.

The consumption of oxygen and carbon dioxide production in fresh-cut pears can be explained in general by the respiration of the tissue, which continues being a living tissue even after cutting, as well as by the respiration of microbial flora which increase along the storage time. A decrease of oxygen levels into the fresh-cut pears package lead to an anaerobic transformation of the pear sugars into alcohol and CO_2 by the native flora, thus increasing the concentration of those gases into the trays as was observed in fresh-cut pears dipped in W (Fig. 3b and 3c). However, a lower consumption of oxygen and lower carbon dioxide and ethanol production through the storage time in those fresh-cut pears dipped in CGLW and CGLW + MA was found, as a consequence of the antimicrobial effect of malic acid and stabilizing substances over the native flora.

Likewise, a significant ($p < 0.05$) increase of ethylene production was found during the storage of fresh-cut pears irrespective of the dipping condition. This patter on ethylene production shows that pears pieces are living tissues that continue ripening after cutting, following a classic behavior of climacteric fruit (Wills *et al.*1998), moreover, during the pear processing a tissue mechanical stress is produce, and in consequence, the production of ethylene by the fruit could be induced (Pech *et al.* 2003). In addition, the ethylene production was influenced by the dipping condition since, smaller amounts of ethylene (Fig. 3d) were found inside the packages of fresh-cut pears dipped in CGLW + MA and CGLW in comparison with those cut-pears dipped in W, reaching concentrations of 14, 12.4 and 5.09 $\mu l \, .l^{-1}$ of ethylene in fresh-cut pears dipped in W, CGLW and CGLW + MA at 14 days of storage, respectively. From that time, ethylene concentration was maintained constant in fresh-cut pears dipped in W, whereas in fresh-cut pears dipped in CGLW slightly decreases (10.27 $\mu l \, .l^{-1}$) and in fresh-cut pears immersed in CGLW + MA continues increasing until 21 days (6.06 µl

$.l^{-1}$) and then decreased (4.47 µl $.l^{-1}$). That fact can also be explained by low oxygen and high carbon dioxide environment reached into the trays. It is widely known that oxygen participates in the biosynthesis of 1-amino-cyclopropane-1-caboxylic acid (ACC) by the action of the ACC synthetase, and serves as co-substrate of the ethylene forming enzyme (EFE) which is the enzyme that transform the ACC in ethylene (Pech *et al.*1990).

Color changes

Storage time had not a significant effect ($p > 0.05$) on the lightness (L*) of fresh-cut pears, while that parameter was significantly affected ($p < 0.05$) by the dipping conditions. Reductions of lightness by dipping conditions or storage time have been reported by others researchers in different variety of fresh-cut pears (Dong *et al.* 2000; Soliva-Fortuny *et al.* 2002a; Gorny *et al.* 2002; Oms-Oliu *et al.* 2006). The maintaining of the color observed through the storage time in this study may attribute to the fruit type and variety used, being advantageous with respect to other pears which should be considerate by researchers and processors. On the other hand, higher values of lightness were found in fresh-cut pears dipped in CGLW or CGLW + MA than in those cut-pears immersed in W (Fig. 4a). Those differences in the initial L* values suggest that a fast darkening occurred in fresh-cut pears dipped in W during the first hours of storage. On the other hand, both, storage time and dipping condition influenced significantly ($p < 0.05$) the hue angle (hº). A significant ($p < 0.05$) decrease of hue angle was observed after 14 days of storage in all dipping conditions, being more noticeable in fresh-cut pears dipped in W (Fig. 4b). On the other hand, higher values of hº during the sample preparation day (t = 0 days) were observed in fresh-cut pears dipped in stabilizing solution with (100.35) or without (99.87) malic acid in comparison with cut-pears dipped in W (90.26) (Fig. 4b). The changes observed in the color of fresh-cut pears may be a result of injury stress caused by processing, which produce a cellular decompartmentalization or delocalization of enzymes and substrates leading to various biochemical deteriorations such as browning, off-flavors, and texture breakdown (Varoquaux and Wiley, 1994).

These results demonstrate the effectiveness of dipping fresh-cut pears in solutions with N-acetyl-L-cysteine and glutathione to control browning as was also reported by Molnar-Perl and Friedman (1990) in apples and potatoes, Rojas-Graü *et al.* (2006a, 2006b) in fresh-cut "Fuji" apple and Oms-Oliu *et al.* (2006) in fresh-cut "Flor de invierno" pear. Molnar-Perl and Friedman (1990) and Richard *et al.* (1991) and Richard-

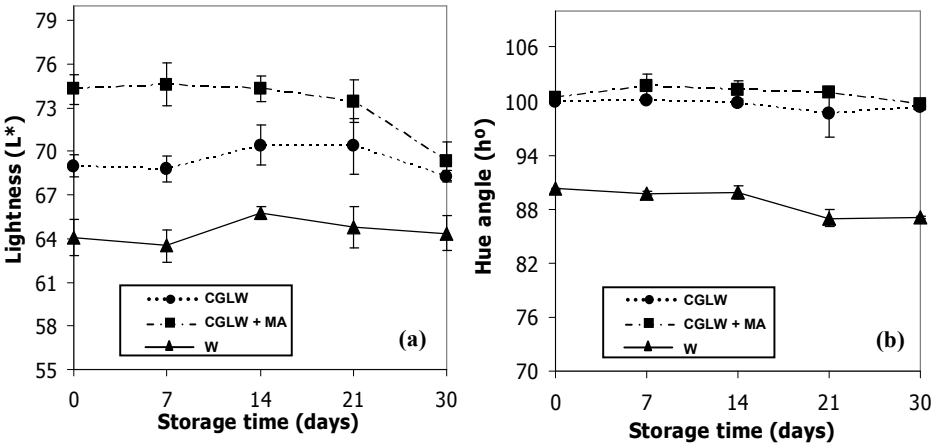

Figure 4.- Changes in lightness (L*) (a) and hue angle (hº) (b) of fresh-cut pears dipped in aqueous solutions of N-acetyl-L-cysteine at 1% (w/v), glutathione at 1% (w/v) and calcium lactate at 1% (w/v) (CGLW), N-acetyl-L-cysteine at 1% (w/v), glutathione at 1% (w/v), calcium lactate at 1% (w/v) and D-L-malic acid 2.5% (w/v) (CGLW + MA) and distilled water as control (W) packed in air and stored at 5ºc during 30 days. The values are mean of 30 determinations ± SD.

Forget *et al*. (1992) indicated that cysteine, N-acetyl-L-cysteine and glutathione can prevent browning by competitive reaction with polyphenol oxidase, reacting with the intermediate quinones to form stable colorless compounds. In addition we found that the incorporation of malic acid to the dipping solution helps to maintain a better color of the fresh-cut pears during storage time, thus demonstrating its antioxidant capacity. Meyer *et al.* (2002) indicated that organic acids as citric, tartaric and malic may also prevent the browning of food by acting as metal chelators.

Firmness changes

Changes in firmness values of fresh-cut pears were negligible throughout the storage time when CGLW or CGLW + MA were used as dipping treatments, instead, when W was used, a decrease from the 7 days of storage was observed (Fig. 5). Rojas-Graü, *et al*. (2006b) found that fresh-cut "Fuji" apple firmness was maintained along the storage time (21

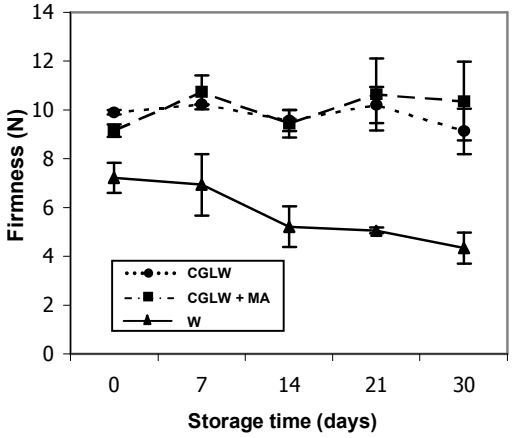

Figure 5.- Changes in firmness (N) of fresh-cut pears dipped in aqueous solutions of N-acetyl-L-cysteine at 1% (w/v), glutathione at 1% (w/v) and calcium lactate at 1% (w/v) (CGLW), N-acetyl-L-cysteine at 1% (w/v), glutathione at 1% (w/v), calcium lactate at 1% (w/v) and D-L-malic acid 2.5% (w/v) (CGLW + MA) and distilled water as control (W) packed in air and stored at 5ºc during 30 days. the values are mean of 10 determinations ± standard deviation.

days), whereas, Soliva-Fortuny *et al.* (2004) found a decrease of the firmness throughout the storage time in fresh-cut "Conference" pears. That difference can be due to type and variety of fruit used as well as microbial spoilage which can be also an influential factor over the fruit firmness, since tissue degradation can occur by action of enzymes (pectic) from microbial origin. Likewise, significant differences ($p < 0.05$) in fresh-cut pears firmness were observed among dipping conditions; showing fresh-cut pears immersed in CGLW and CGLW + MA higher values of firmness than those immersed in W at the sample preparation day (t = 0 day) (Fig. 5). Differences in firmness between untreated and treated with calcium fresh-cut pears were also found by Dong *et al*. (2000).

Differences among dipping conditions in fresh-cut pears firmness could be due to physical and mechanical alterations occurred during the first hours of storage in the tissue of fresh-cut pears dipped in W as a consequence of changes in the chemical structure of the cell wall polysaccharides. This fact demonstrates the high influence of the dipping treatment in solutions with calcium lactate (CGLW and CGLW + MA) over the prevention of softening of fresh-cut pears. Calcium plays a special role in maintaining the cell wall structure by interacting with the pectic acid to form calcium pectate, and is highly recommendable to minimize physiological disorders (Poovaiah, 1986). Calcium chloride and calcium lactate have been used as firming agents in cut apples, pears and melon with good results (Ponting *et al.* 1972; Luna-Guzmán and Barret, 2000; Gorny *et al.* 2002; Soliva-Fortuny *et al.* 2002b, 2004 and 2002c; Rojas-Graü *et al.* 2006b) however, some investigators have indicated that cut-fruit treated with calcium lactate showed better flavor than those treated with calcium chloride (Ponting *et al.* 1972; Luna-Guzmán and Barret, 2000).

Sensory evaluation of fresh-cut pears

Statistically significant differences ($p < 0.05$) in odor, color, firmness, taste and acidity between the same dipping treatment conditions were not detected by the panelists at 0 and 15 days of storage at 5ºC.. However, significant differences ($p < 0.05$) in fresh-cut pears color dipped in W and those dipped in CGLW and CGLW + MA were detected from the same day of the samples preparation (t = 0 day), being the better accepted by panelists those fresh-cut pears dipped in CGLW and CGLW + MA (Fig. 6). Sensory results are in accordance with those found by instrumental determination, since, lower lightness (L*) and hº values were found in fresh-cut pears dipped in W in comparison with those found in fresh-cut pears immersed in CGLW + MA and CGLW from the sample preparation day (Fig. 4). In contrast, firmness differences among dipping conditions were not detected by the panelists (Fig. 6), whereas instrumentally those differences were found (Fig. 5). On the other hand, the taste and acidity of fresh-cut pear dipped in CGLW + MA at 0 or 15 days received the higher scores (Fig.6). In general, fresh-cut pear dipped in CGLW + MA and CGLW exhibited a major acceptation by panelists.

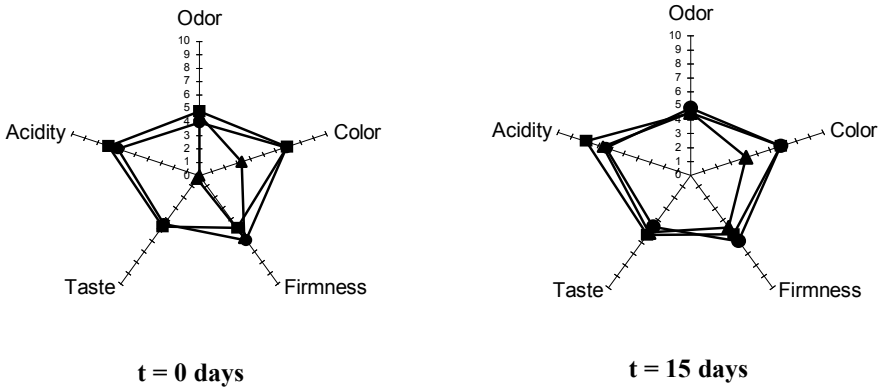

Figure 6.- Influence of the storage time on sensory characteristic of fresh-cut pears dipped in aqueous solutions of N-acetyl-L-cysteine at 1% (w/v), glutathione at 1% (w/v) and calcium lactate at 1% (w/v) (CGLW), N-acetyl-L-cysteine at 1% (w/v), glutathione at 1% (w/v), calcium lactate at 1% (w/v) and D-L-malic acid 2.5% (w/v) (CGLW + MA) and distilled water as control (W) packed in air and stored at 5ºc during 15 days. The values are mean of 30 determinations. taste and acidity determination at 15 days in fresh-cut pears dipped in w were not carried out due to its high microbiological load.

CONCLUSIONS

Fresh-cut pears immersed in an aqueous solution of N-acetyl-L-cysteine (1%), glutathione (1%), calcium lactate (1%) and malic acid (2.5%) resulted to be effective to reduce pathogenic populations of *L. monocytogenes, S.* Enteritidis and *E. coli* O157:H7 by more than 5 log cycles. In addition, an extension of the fresh-cut pears shelf-life from both physicochemical and microbiological point of view without significantly affect their sensory attributes was also achieved, representing a good and practical alternative for fresh-cut fruit processing.

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Comparative study on essential oils and their active compounds incorporated in alginate-based edible coating to assure the safety and quality of fresh-cut Fuji apples

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ABSTRACT

The effectiveness of cinnamon, clove and lemongrass oils as well as their active compounds cinnamaldehyde, eugenol and citral as antimicrobial agents incorporated in combination with malic acid and quality stabilizing such as N-acetyl-L-cysteine, glutathione and calcium lactate into alginate-based edible coating to assure the safety and quality of fresh-cut Fuji apples was compared. Edible coating containing malic acid and quality stabilizing substance (EC) applied on apple pieces was effective to maintain the physicochemical characteristics (odor, color, firmness, taste and acidity) by more than 30 days, decrease the respiration rate, reduce inoculated *E. coli* O157:H7 population about 1.23 Log CFU/g at t = 0 days and extend the microbiological shelf-life by at least 19 days. However, the incorporation of essential oils (EOs) at 0.7% (v/v) or their active compounds at 0.5% (v/v) into EC, increased even more their antimicrobial effects, since more than 4 Log CFU/g reductions of *E. coli* O157:H7 population and an extension of the microbiological shelf-life by more than 30 days were achieved. In contrast, physicochemical characteristics in fresh-cut apples coated with EC + EOs in high concentrations or their active compounds were affected, limiting their shelf-life between 7 and 21 days. Among the selected EOs and active compounds, lemongrass and cinnamon oil (0.7% v/v), citral (0.5% v/v) and cynnamaldehyde (0.5% v/v) were the most effective from a microbiological point of view, whereas EOs of lemongrass, cinnamon and clove at 0.3% (v/v) better maintained the physicochemical characteristics. In addition, apple pieces with EC were sensorially preferred by the panelists at t = 0 days, whereas apple pieces with EC and EC + lemongrass at 0.7% (v/v) were the preferred at t = 15 days.

Key words: Antimicrobials, malic acid, essential oils, alginate-based edible coating, fresh-cut apple, *E. coli* O157:H7, shelf-life.

INTRODUCTION

The production of fresh-cut fruits has significantly increased in the last years, presumably due, in part, to their fresh-like characteristics, low caloric content, convenience and an active promotion of fruits and vegetables as basic components of a healthy diet. Nevertheless, it is well known that some minimal processing may alters the integrity of the fruit and induce surface damages increasing the tissue respiration and leading to biochemical deteriorations such as browning, off-flavor development and texture breakdown, which can damage the fresh-cut fruit quality (*26, 29, 36, 45, 51*). In addition, microbial contamination in fresh-cut fruits could also occur by transference of naturally occurring microorganisms from skim to flesh, increasing the fruit spoilage and the risk for consumer due to the pathogenic microorganisms presence. In such sense, *E. coli* O157:H7 which is an enteric pathogen that can cause hemorrhagic colitis and hemolytic uremic syndrome (*30*) has been associated with several outbreaks of foodborne illness caused by consumption of apple juice and apple cider in recent years (*3, 8, 18, 60*). In addition, survival and growth of this pathogenic microorganism in fresh-cut apples as well as in apple cider have been also demonstrated (*9, 18, 31*).

The effectiveness of different antimicrobial substances such as lysozyme, nisin, organics acids, essential oils (EOs) and their derivatives incorporated into the edible films against several pathogens have showed to be satisfactory (*7, 11, 39, 40, 43, 48*). However, very few studies have been published about effectiveness of those compounds incorporated into edible coatings for ensuring the quality and safety on fresh-cut fruits (*12, 50*).

Edible coatings can act as moisture and gas barriers preventing the moisture and aroma loss of the fresh-cut fruit, delaying color changes and maintaining the general appearance of the product through the storage time (*35*). But also, they can operate as carriers of food ingredients including antibrowning agents, colorants, flavors, nutrients, spices and antimicrobials (*14, 28*) for avoiding microbial spoilage, extending the shelf-life and enhancing the safety of those fruit products (*7, 14, 39, 50*). For these reasons, the use of edible coatings in a wide range of food products, including fresh and minimally processed fruits, is receiving great interest by food industry.

Substances selected for this study such as malic acid, stabilizing substances (calcium lactate, glutathione, cysteine), alginate, EOs (cinnamon, clove, lemongrass) and their active compounds

(cinnamaldehyde, eugenol, citral) are considered as Generally Recognized as Safe (GRAS) according to Food Additive Status List (*61*) and some of them have been used as preservative, antioxidant and flavoring agents (*6, 15, 44, 45, 50, 59*). However, the use of a combination of those substances incorporated into edible alginate-based coating to inactivate pathogenic microorganisms such as *E. coli* O157:H7 and assure the quality of fresh-cut apples has not been still studied.

The objective of this study was to compare the effectiveness of cinnamon, clove and lemongrass oils as well as their active compounds cinnamaldehyde, eugenol and citral as antimicrobial agents incorporated in combination with malic acid and quality stabilizing such as N-acetyl-L-cysteine, glutathione and calcium lactate into alginate-based edible coating to assure the safety and quality of fresh-cut Fuji apples.

MATERIALS AND METHODS

Fruit and flesh characterization. "Fuji" apples (*Malus domestica* Borkh), at commercial ripeness stage were provided by ACTEL, Lleida, Spain. Total acidity, expressed as g of malic acid /100 ml, pH (Crison 2001 pH-meter; Crison Instruments S. A., Barcelona, Spain) and soluble solids content (%) (Atago RX-1000 refractometer; Atago Company Ltd, Japan) were measured according to the official methods of fruit juices and other vegetables and derivatives (*4*). Firmness (TA-TX2 Texture Analyzer, Stable Micro Systems LTD, Surrey, England) and color (Tri-stimulus Minolta CR-400 Chroma Meter, Konica Minolta Sensing, INC, Osaka, Japan) were also measured to characterize the fruit (Table 1).

Table 1. Physicochemical characteristics of apple flesh

Total acidity (g malic acid/100 ml)	0.34 ± 0.03
pH	4.33 ± 0.02
Soluble solids content (%)	11.98 ± 0.31
Firmness (N)	10.3 ± 0.6
Color	L* = 74.24 ± 0.07
	a* = -1.10 ± 0.05
	b* = 25.01 ± 0.25

Edible coating solutions. Edible coating were elaborated from a base-solution of alginate (Food grade sodium alginate, Keltone LV, ISP, San Diego, CA., USA) at 2% (w/v) and glycerol (Merck, Whitehouse Station, N.J., USA) at 1.5% (v/v) which were prepared into sterile distilled water previously heated at 70ºC and stirred until total dissolution of the components. Essential oils (EOs) from Cinnamon bark (*Cinnamomum zeylanicum*), Clove (*Eugenia caryophyllata*) and lemongrass (*Cymbopogon citrates*) were added to edible coating solution at 0.3 and 0.7% (v/v); whereas, their main active compounds cynamaldehyde, eugenol and citral were incorporated into the coating base solution at 0.5% (v/v). The EOs and their compounds were purchased to Aceites Esenciales Dicana (Barcelona, Spain). Edible coating without essential oil, as control, was also evaluated. Those solutions were then mixed for 3 min using an Ultra Turrax T25 homogenizer (IKA® WERKE, Germany) with a S25N-G25G device, and subsequently degassed through a vacuum pump (Vaccubrand, Wertheim, Germany) to eliminate burbles that may affect the homogeneity of the coating and can cause breaking of it. In addition, a bath with aqueous solution containing 1% (w/v) N-acetyl-L-cysteine (Acros Organics, New Jersey, USA) and 1% (w/v) glutathione (Acros Organics) as antibrowning substances, 2% (w/v) calcium lactate pent-hydrate (Scharlau Chemie, S.A. Barcelona, Spain) as anti-softening agent and 2.5% (w/v) D-L malic acid (Scharlau Chemie, S.A.) as antimicrobial, dissolved in distilled water were used to crosslink the carbohydrate polymers in the edible coating. The concentrations selected in this work were based on previous studies (*44, 45, 50, 53*). Base-solution of edible coating without essential oils or active compounds and cross-linked to malic acid and stabilizing substances was defined as EC.

Strain and inoculum preparation. A strain of *E. coli* O157:H7 (CECT 4267) from the Spanish Type Culture Collection (University of Valencia, Valencia, Spain) isolated from a sample of human feces from outbreak of hemorrhagic colitis occurred in Oregon, USA, was used in this study. The strain was maintained in Tryptone soy agar (TSA) (Biokar Diagnostics, Beauvais, France) slants at 5ºC. Stock culture of *E. coli* O157:H7 was grown in tryptone soy broth (TSB) (Biokar Diagnostics) plus 0.6% (w/v) yeast extract (Biokar Diagnostics) at 37ºC for 11 hours and 120 rpm to obtain cells in early stationary phase. The maximum level reached for the microorganism was 3.8 x 10^9 colony forming units/ milliliter (CFU/ml). Concentration was then adjusted to 10^8 (CFU/ml) using saline

peptone water (0.1% peptone, Biokar Diagnostics, plus 0.85% NaCl, Scharlau Chemie, S.A.).

Apples processing and packaging. Apples were sanitized by immersion in sodium hypochlorite solution (300 ppm, pH 6.8) for 2 min; then washed with potable water and finally dried with absorbent paper. The cleaned fruit were cut in the extremes with a knife, and then with a cylindrical hollow instrument of stainless steel into pieces of 1.4 cm diameter x 2.0 cm height (approximately 6 cylinders per apple). Ten apple pieces with a total weight of 50 g were placed into the polypropylene trays and then inoculated uniformly by spreading 500 µl of *E. coli* O157:H7 stock culture (10^8 CFU/ml) over its entire upper surface with a sterile micropipette for challenge study. After thirty minutes of being inoculated, apple pieces were dipped into coating solutions for 2 min at 18ºC, afterwards residual solution was allowed to drip off for 1 min, just before submerging the apple pieces for additional 2 min in the solution of calcium lactate at 18ºC to favors the crosslinking. The trays were then wrap-sealed with a 64 µm thick polypropylene film of water vapor permeability of 142.86 fmol $s^{-1}m^{-2}$ kPa^{-1} at 38ºC and 90% R.H., O_2 permeability of 52.38 fmol $s^{-1}m^{-2}$ kPa^{-1} at 23ºC and 0% R.H., and CO_2 permeability of 2.38 fmol s^{-1} m^{-2} kPa^{-1} at 23ºC and 0% R.H. (Tecnopack SRL, Mortara, Italy) using a vacuum compensated packaging machine (ILPRA Food Pack Basic V/6, Ilpra S. CP. Vigevono, Italy). Trays containing inoculated apple pieces coated or non-coated (control) were sealed with ordinary atmosphere (air) and stored in refrigeration at 5ºC for 30 days. In addition, 50 g of uninoculated apple pieces coated or non-coated were packed as previously described and stored at 5ºC for 30 days for shelf-life and sensory studies.

A total of 12 trays of coated apple pieces inoculated with *E. coli* O157:H7 were prepared by each tested antimicrobial and analyzed in duplicated at 0, 3, 7, 14, 21 and 30 days of storage, whereas 12 trays of non-coated fresh-cut apple pieces inoculated with *E. coli* O157:H7 were prepared as control samples. In addition, a total of 22 trays with non-inoculated apple pieces coated by each tested antimicrobial were prepared for shelf-life estimation (10 trays for physicochemical analysis and 12 trays for microbiological analysis), whereas 12 trays of coated uninoculated apple pieces were prepared by selected antimicrobial to carry out a sensory evaluation at 0 and 15 days of storage.

Microbiological analysis. Enumeration of *E. coli* O157:H7 in inoculated apple pieces non-coated or coated were carried out at 0, 3, 7, 14,

21 and 30 days of storage from an initial dilution of the sample (50 g of sample in 450 ml of buffered peptone water (BPW) pH 7.2, Biokar Diagnostics) using a masticator (IUL Instruments, Barcelona, Spain) during 1 min and preparing seriated dilutions which were spread at reason of 0.1 ml in McConkey-sorbitol agar (Biokar Diagnostics) plates. Those plates were incubated at 37ºC for 24 h. A pair of trays with inoculated fresh-cut apples coated or non-coated were randomly chosen and evaluated in duplicated at each established time.

Counts of mesophilic, psychrophilic and yeasts and molds populations in coated apple pieces non-inoculated were made at 0, 3, 7, 14, 21 and 30 days, in order to evaluate the influence of the malic acid, EOs and their main active compounds incorporated into edible coating solutions over those populations. A pair of trays with coated fresh-cut apples non-inoculated were also randomly chosen and analyzed in duplicated at each indicated time. Mesophilic and psychrophilic microorganisms counts were made according to the ISO 4833:1991 (*19*) guideline using plate count agar (PCA) (Biokar Diagnostics) and pour plate method. Plates of psychrophilic microorganisms were incubated at 5ºC for 10-14 days, whereas, mesophilic microorganisms were incubated at 35ºC for 24-48 h. On the other hand, yeasts and molds counts were made according to the ISO 7954:1987 (*20*) guideline using chloramphenicol glucose agar (CGA) (Biokar Diagnostics) and spread plate method. Plates were incubated at room temperature (18ºC) for 3-5 days. All the microbial counts were expressed as Log_{10} CFU/g and reported values are the mean of two determinations made in duplicate ± standard deviation (n = 4).

Headspace gases analysis. The gas composition of the package headspace was determined using a Micro-GP CP 2002 gas analyzer (Chrompack International, Middelburg, Netherlands) equipped with a thermal conductivity detector. A sample of 1.7 ml was automatically withdrawn from the headspace atmosphere. Portions of 0.25 and 0.33 µl were injected for O_2 and CO_2 determination, respectively. The O_2 content was analyzed with a CP-Molsieve 5Å packed column (Chrompack International) (4m x 0.32mm, df =10mm) at 60ºC and 100 kPa. On the other hand, a Pora-PLOT Q column (Chrompack International) (10m x 0.32mm, df =10mm) was held at 70ºC and 200 kPa for CO_2, ethylene, and ethanol quantification. A pair of trays with coated fresh-cut apples non-inoculated for each edible coating condition and uncoated control sample were randomly taken and analyzed in duplicated at 0, 7, 14, 21 and 30 days

of storage. Thus, the reported values are the mean of two determinations in duplicate ± standard deviation (n = 4).

Firmness determination. Measurement of firmness was performed in ten pieces by each pair of trays for each edible coating condition and uncoated control sample, using a TA-TX2 Texture Analyzer (Stable Micro Systems LTD. Surrey, England) and the following conditions: Pre-test speed: 2 mm/s, test speed: 5.0 mm/s, post-test speed: 5.0 mm/s and penetration distance: 10 mm. The resistance of penetration was measured as the strength necessary for a cylindrical probe of 4 mm of diameter and plane basis to penetrate a cylindrical sample of apple flesh of 2.00 cm height. Five pieces of each tray were analyzed; therefore, the reported values are the mean of 10 determinations ± standard deviation.

Color determination. Color of fresh-cut apples was determined in 10 pieces from a pair of trays by each edible coating condition and uncoated control sample randomly chosen using a tri-stimulus Minolta CR-400 Chroma Meter (Konica Minolta Sensing, INC. Osaka, Japan) with the illumining D75 and observation angle of 10º, calibrated with a standard white plate (Y = 94.00, x = 0.3158, y = 0.3322). Three readings of L* (Lightness), a* (green chromaticity) and b* (yellow chromaticity) coordinates were recorded for each apple piece. Numerical values of a* and b* parameters were employed to calculated hue angle (hº) through the equations 1:

$$h^{o} = \arctan \frac{b^{*}}{a^{*}} \qquad (1)$$

The reported values are the mean of thirty determinations ± SD.

Sensory evaluation. Fresh-cut apples non-coated, coated with EC without EOs and coated with EC + EOs of cinnamon, clove or lemongrass at 0.7% (v/v) were used to carry out a sensory analysis. Pieces of apples prepared the same day of the test (t = 0) and also those stored at 5ºC for 15 days were evaluated by thirty volunteers aged between 20-50 years old, who like and eat apple frequently. A total of 10 samples (5 prepared at t = 0 days and 5 stored for 15 days) labelled with 3 digit code numbers were randomly provided to the panellists. The evaluated samples by each time comprised three samples with EC including EOs, one sample with EC without EOs and one sample of fresh-cut apples non-coated. A glass containing potable water and pieces of non-salted cracker were provided to

panellists for eliminating the residual taste between samples. Odour, color, taste, and firmness were the evaluated attributes by the panellists. Each attribute was scored on a structured hedonic scale labelled from "extremely unpleasant" (0) to "extremely pleasant" (10).

Data modeling and statistical analysis. Statistical package Statgraphics Plus version 5.1 was used to compare applied treatments through an analysis of variance (ANOVA) with posterior multiple range test (MRT) as well as to model the microbiological data. ANOVA with MRT were applied to find significant differences ($p < 0.05$) among storage time and edible coating condition on microbiological counts, headspace gas composition, firmness, color and sensory evaluation profile. Mesophilic, psychrophilic and yeasts and molds growth in coated fresh-cut apples non-inoculated were modeled according to the modified Gompertz's equation by Zwietering *et al*. (*63*) (Equation 2). In addition, microbiological shelf-life (***SL***) was calculated through equation (3) obtained by rearranged of the modified Gompertz's equation, considering as maximum limit 10^7 CFU/g of mesophilic total count at expiry date in accordance with the Spanish regulation for hygienic processing, distribution and commerce of prepared meals (*5*).

$$Y = k + A \cdot exp\{-exp[(\mu_{max} \cdot 2.7182 / A)(\lambda - t) + 1]\} \quad (2)$$

$$SL = \lambda - \frac{A\left\{ln\left[-ln\left(\frac{log(10^7) - k}{A}\right)\right] - 1\right\}}{\mu_{max} \cdot 2.7182} \quad (3)$$

where *Y*, is the count of microorganisms (log_{10} CFU/g) for a given time; *k*, is the microorganism initial count estimated by the model (log_{10} CFU/g); *A*, is the maximum microorganism growth attained at the stationary phase (log_{10} CFU/g); μ_{max}, is the maximal growth rate [Δ log_{10} (CFU/g)/day]; λ, is the lag time (days), *t,* is the storage time (days) and *SL*, is the microbiological shelf-life time.

On the other hand, shelf-life from a color and firmness point of view was estimated basing on established reference point in the laboratory from a

comparison of apple pieces showing undesirables characteristics and fresh-cut apples.

RESULTS AND DISCUSSION

Reduction of *E. coli* O157:H7 population. Alginate-based edible coating containing malic acid without EOs (EC) resulted effective against *E. coli* O157:H7 population in fresh-cut apples, since, an reduction of 1.23 Log of its population at the samples preparation day (t = 0 days) was observed in comparison with the non-coated apple pieces (Table 2). However, a higher effectiveness against this pathogenic microorganism was observed when EOs or their actives compounds were incorporated into the coating, indicating that an enhanced antimicrobial effect was caused by the combined incorporation of malic acid and EOs into the coating. In general, higher concentrations of EOs were more powerful than those lower concentrations, being lemongrass (0.7% v/v) the most effective against *E. coli* O157:H7 among the EOs studied (Table 2), since, up to 4.02 Log reductions of *E. coli* O157:H7 were found just after applying the edible coating (t = 0 days). Nychas *et al*., (*34*) indicated that the mode of action of each EO depends on its concentration, where lower concentrations may inhibit enzymes associated with energy production, while higher concentrations may precipitate proteins, being this latter consequence the most effective for inactivation of microbial cells. Likewise, citral (0.5% v/v) among the active compounds exhibited a stronger antimicrobial effect against *E. coli* O157:H7 at t = 0 days than cinnamaldehyde (0.5% v/v) and eugenol (0.5% v/v) (Table 2).

The results indicated that lemongrass oil and its main active compound citral acted faster against *E. coli* O157:H7 at day 0 than cinnamon and clove oil or their active compounds, cinnamaldehyde and eugenol, suggesting that lemongrass oil and citral could pass to cell inside more easily causing irreversible damages to the bacterial cell, which induced the microorganism death. This fact could be due to the citral partition coefficient (2.8-3.0 $LogP_{ow}$ (*37*)), which is higher than those reported for cinnamaldehyde (2.22 $LogP_{ow}$ (*21*)) and eugenol (2.27 $LogP_{ow}$ (*27*)), favoring its diffusion through the cell membrane. Burt (*6*) reported that the hydrophobicity is an important characteristic of EOs, which make them enables to entry in the cell membrane and mitochondria, disturbing the

Table 2.- Survival fraction of *E. coli* O157:H7 in inoculated fresh-cut apples coated with EC including or not EOs or their active compounds and stored at 5ºC for 30 days.

Treatment	Survival fraction [a] (Log_{10} CFU/g)					
	Storage time (days)					
	0	3	7	14	21	30
Non-coated	6.26 ± 0.12Aa	5.75 ± 0.02Ab	5.31 ± 0.21Ac	4.90 ± 0.22Ac	3.78 ± 0.25Ad	3.49 ± 0.10Ad
EC	5.03 ± 0.06Ba	4.7 ± 0.4Bab	3.872 ± 0.01Bbc	3.3 ± 0.8Bcd	2.92 ± 0.02Bcf	2.15 ± 0.21Bf
EC + Cin (0.3%)	4.11 ± 0.07Ca	3.3 ± 0.1CDb	2.8 ± 0.5CDb	2.17 ± 0.08Cc	< 2Cc	< 2BCc
EC + Cin (0.7%)	3.14 ± 0.06Fa	< 2Fb	< 2Eb	< 2Cb	< 2Cb	< 2BCb
EC + Cy (0.5%)	3.26 ± 0.05Ea	< 2Fb	< 2Eb	< 2Cb	< 2Cb	< 2BCb
EC + Clo (0.3%)	3.88 ± 0.06Da	3.35 ± 0.07CDab	2.99 ± 0.03Cbc	2.51 ± 0.21Cd	2.5 ± 0.1BCcd	< 2Cd
EC + Clo (0.7%)	2.89 ± 0.16Ga	2.63 ± 0.21Ea	< 2Eb	< 2Cb	< 2Cb	< 2BCb
EC + Eu (0.5%)	3.36 ± 0.08Ea	3.11 ± 0.0 3DEa	2.4 ± 0.3DEb	2.2 ± 0.3Cb	< 2Cb	< 2BCb
EC + Lem (0.3%)	4.16 ± 0.01Ca	3.68 ± 0.14Ca	2.7 ± 0.3CDb	2.5 ± 0.7Cb	2.19 ± 0.28Cb	< 2BCb
EC + Lem (0.7%)	2.24 ± 0.09Ia	< 2Fb	< 2Eb	< 2Cb	< 2Cb	< 2BCb
EC + Cit (0.5%)	2.65 ± 0.07Ha	< 2Fb	< 2Eb	< 2Cb	< 2Cb	< 2BCb

[a] Values are the mean of 4 determinations ± standard deviation.
EC = Edible coating; Cin = Cinnamon; Cy = Cinnamaldehyde; Clo = Clove; Eu = Eugenol; Lem = Lemongrass; Cit = Citral.
Values in the same column that are not followed by the same capital letter are significantly different ($p < 0.05$); values in the same row that are not followed by the same lower-case letter are significantly different ($p < 0.05$).

structures and rendering them more permeable. Nikaido (*33*) reported that all Gram negative bacteria possess an outer membrane (OM) which provides the bacterium with a hydrophilic surface due to the presence of lipopolysaccharide molecules, also indicating, that small hydrophilic solutes are able to pass the OM through abundant porin proteins provided of hydrophilic transmembrane channels, whereas that OM serve as a penetration barrier toward macromolecules and to hydrophobic compounds. However, those authors point out that the OM is not totally impermeable to low mass weight hydrophobic molecules, and thus, some of them can slowly pass across porins.

Different action mechanisms could explain the bactericidal effects caused by the EOs selected for this study. Thus, disruption of the cell membrane with alteration of ATPasa activity and leakage of cellular constituents could justify the action mode of lemongrass oil, clove oil, and their active compounds citral and eugenol, whereas reduction of the

intracellular ATP and inhibition of important enzymes without significant disruption of the cell membrane for cinnamon oil and cinnamaldehyde could be suggested. In such sense, Lambert *et al*., (*23*), Gill and Holley (*16, 17*) and Oussalah *et al*., (*38*) reported that oregano oil, savory oil, thymol, eugenol and carvacrol caused disruption of the cellular membrane, inhibition of the ATPase activity and release of the intracellular ATP of *E. coli*, *E. coli* O157:H7, *L. monocytogenes*, *Lactobacillus sakei*, *Pseudomonas aeruginosa* and *Sthaphylococcus aureus*. Likewise, disruption of the membrane with posterior leakage of cell content in *S.* Enteritidis cell cultured in apple juice for 24 at 35C was reported by Raybaudi-Massilia et al., (*44*). In contrast, Oussalah *et al* (*38*) and Gill and Holley (*16, 17*) reported a decrease in the intracellular ATP by ATPase activity without apparent changes on the cell membrane of *E. coli*, *E. coli* O157:H7 and *L. monocytogenes* when cinnamon oil or cynnamaldehyde were used. Those authors suggested that a mechanism of action for cynnamaldehyde in which interaction with the cell membrane causes disruption sufficient to disperse the proton motive force by leakage of small ions without leakage of larges cell component such as ATP could occur. In addition, Wendakoon and Sakaguchi (*62*) mentioned an action possible of cynnamaldehyde on the embedded proteins in the cytoplasm membrane of *Enterobacter aerogenes* by inhibition of the enzymes amino acid decarboxylases, which are necessary for the amino acid biosynthesis and biodegradation.

Meaningful decreases ($p < 0.05$) of *E. coli* O157:H7 population through the storage time in non-coated and coated fresh-cut apples were also observed, being this reductions more drastic when EOs in high concentrations or their active compounds were added to the coating (Table 2). Reductions of this pathogen in control samples suggests that effects of the storage temperature (5ºC) and competitive flora (native flora) through the storage time could affect the survival of *E. coli* O157:H7 inoculated in apple pieces since, a reduction of its population from 6.26 ± 0.12 to 3.49 ± 0.10 Log_{10} CFU/g after 30 days of storage at 5ºC was observed. A similar behavior was reported by Fisher and Golden (*13*), who observed a slight reduction of *E. coli* O157:H7 population in fresh-cut Golden delicious, Red delicious, Winesap and Rome apples during 18 days at 4ºC. On the other hand, among the selected EOs and active compounds, lemongrass and cinnamon oil (0.7% v/v), cynnamaldehyde (0.5% v/v) and citral (0.5% v/v) reduced *E. coli* O157:H7 populations more quickly than clove oil and eugenol during the storage time, since populations of that microorganism

were not detected from 3 days of storage in those fresh-cut apples coated with EC including those substances (Table 2).

Although, studies about the effects of EOs or their active compounds incorporated in edible coatings against *E. coli* O157:H7 inoculated in fresh-cut apples were not found in the literature, the effects of EOs over intentionally inoculated microorganisms used as pathogen indicators in fresh-cut fruits have been reported. Hence, Lanciotti *et al*., (*24*) reported that the addition of 0.02% (v/v) of citrus, mandarin, cider, lemon and lime oils to fresh sliced fruit mixture (apple, pear, grape, peach and kiwifruit) increased the death rate of *E. coli* population. Likewise, Rojas-Graü *et al*., (*50*) indicated that the incorporation of EOs such as lemongrass (1.0-1.5% v/v) and oregano (0.1-0.5% v/v) into an apple puree-alginate edible coating significantly reduced the population of *L. innocua* in fresh-cut apples.

Effects on the native microflora. Significant reductions ($p < 0.05$) of mesophilic, psychrophilic and yeasts and moulds populations in fresh-cut apples coated with EC at t = 0 days in comparison with non-coated apple pieces did not occur (Fig. 1), indicating that initial native flora of apple pieces could be predominantly constituted by acid tolerant microorganisms as a consequence of the pH of the apples, thus being, not immediately affected by the malic acid added to the coating. However, those native populations were significantly affected ($p< 0.05$) by the incorporation of EOs in high concentrations or their active compounds into the coating, since, they were not detected after processing of the apple pieces (t = 0 days) (Fig. 1). Similar results were found by Rojas-Graü *et al*., (*50*) in fresh-cut apples coated with an apple puree-alginate edible coating containing essential oils of lemongrass (1 and 1.5% v/v) and oregano (0.5% v/v).

On the other hand, microbial growth modeling of native microflora in fresh-cut apples along the storage time by the modified Gompertz's equation by Zwietering *et al*. (*63*) (Eq. 2) allowed to detect the inhibitory effects of malic acid alone or in combination with EOs or their active compounds over the growth of mesophilic, psychrophilic and yeasts and molds populations (Fig. 1). Tables 3 shows mean values of the Gompertz's parameters that described the microbial growth of native flora in coated and non-coated fresh-cut apples.

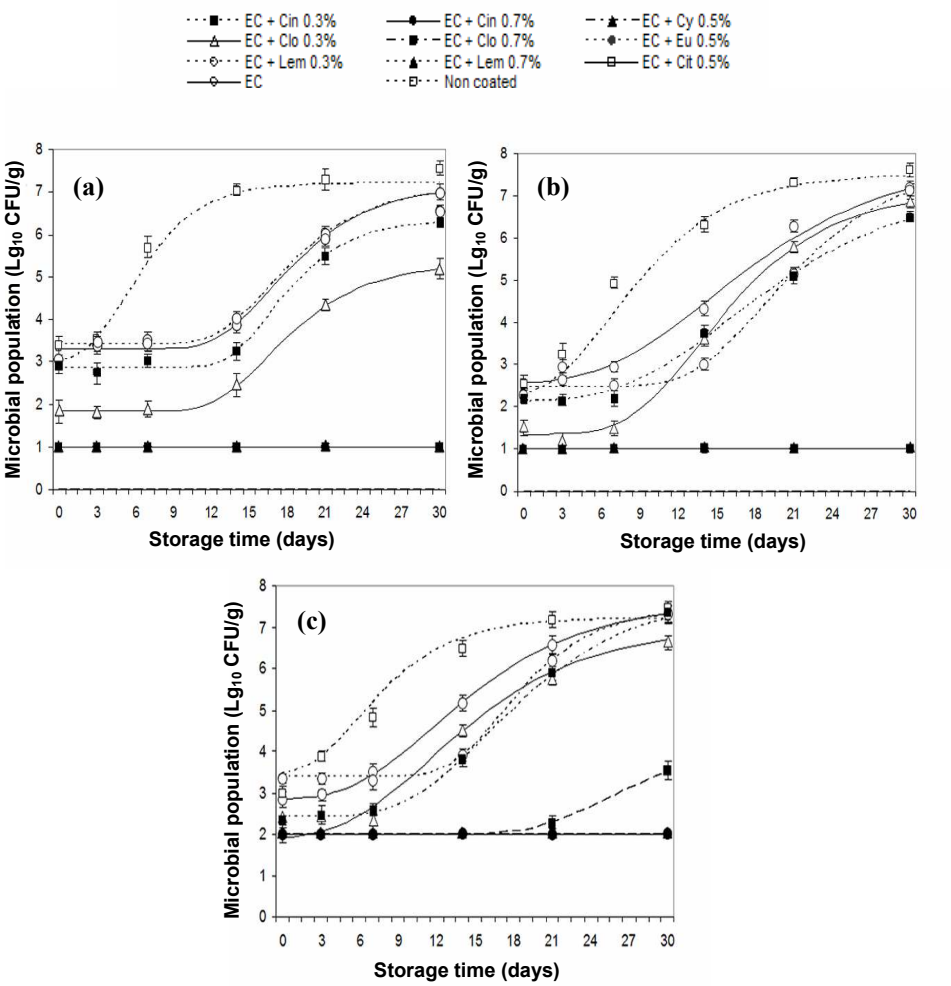

Figure 1.- Growth of mesophilic (a), psychrophilic (b) and yeast and molds (c) in non-inoculated fresh-cut apples coated with alginate-based edible coating (EC) including or not EOs of cinnamon (Cin), clove (Clo), lemongrass (Lem) at 0.3 and 0.7% (v/v) or their active compounds cinnamaldehyde (Cy), eugenol (Eu) and citral (Cit) at 0.5% (v/v). Points represented with geometrical figures indicate values observed, whereas lines represent fitted curves by Gompertz modified equation. The values represented are the mean of 4 determinations ± standard deviation. Detection limits 1 Log_{10} CFU/g.

Table 3.- Gompertz parameters that describe the growth of mesophilic, psychrophilic and yeast and molds in fresh-cut apples coated with EC including or not EOs and stored at 5ºC for 30 days.

Population	Treatment	R^2	MSE	Gompertz parameters[a]				Shelf-life[b] (days)
				k	A	μ_{max}	λ	
Mesophilic	**Non-coated**	98.40	0.13	3.03 ± 0.16	4.18 ± 0.19	0.49 ± 0.11	2.2 ± 0.64	12.26
	EC	97.92	0.06	3.31 ± 0.09	3.85 ± 0.24	0.33 ± 0.05	12.4 ± 0.6	27.12
	EC + Cin (0.3%)	95.70	0.13	2.87 ± 0.15	3.5 ± 0.4	0.38 ± 0.09	13.3 ± 1.3	> 30
	EC + Clo (0.3%)	99.30	0.019	1.83 ± 0.05	3.50 ± 0.15	0.30 ± 0.03	12.6 ± 0.6	> 30
	EC + Lem (0.3%)	99.01	0.03	3.41 ± 0.07	3.72 ± 0.19	0.32 ± 0.04	12.4 ± 0.7	27.77
Psychrophilic	**Non-coated**	96.10	0.22	2.21 ± 0.06	5.3 ± 0.1	0.4 ± 0.2	2.0 ± 0.5	14.09
	EC	98.83	0.06	2.56 ± 0.16	5.3 ± 0.6	0.24 ± 0.03	6.1 ± 1.7	21.46
	EC + Cin (0.3%)	94.99	0.22	2.13 ± 0.27	5.2 ± 1.4	0.23 ± 0.07	7.6 ± 3.3	> 30
	EC + Clo (0.3%)	98.25	0.13	1.36 ± 0.17	5.8 ± 0.5	0.38 ± 0.08	8.1 ± 1.7	30
	EC + Lem (0.3%)	99.60	0.02	2.47 ± 0.05	5.34 ± 0.28	0.340 ± 0.025	13.0 ± 0.5	24.55
Yeast and molds	**Non-coated**	99.30	0.03	3.42 ± 0.30	3.8 ± 0.6	0.35 ± 0.05	2 ± 2.12	14.31
	EC	99.53	0.021	2.83 ± 0.10	4.78 ± 0.23	0.27 ± 0.02	5.2 ± 0.9	19.55
	EC + Cin (0.3%)	96.34	0.20	2.42 ± 0.19	5.4 ± 0.9	0.31 ± 0.08	9.55 ± 2.18	22.45
	EC + Clo (0.3%)	98.53	0.08	1.78 ± 0.21	5.10 ± 0.46	0.28 ± 0.04	5.7 ± 1.7	> 30
	EC + Clo (0.7%)	99.00	0.003	1.990 ± 0.019	1.33 ± 0.10	0.17 ± 0.03	19.35 ± 0.01	> 30
	EC + Lem (0.3%)	98.77	0.05	3.39 ± 0.08	4.09 ± 0.24	0.37 ± 0.05	12.93 ± 0.74	22.39

[a] Data obtained from modified Gompertz model (Eq. 2)
[b] Shelf-life obtained from modified Gompertz model (Eq. 3)
EC = Edible coating; Cin = Cinnamon; Clo = Clove; Lem = Lemongrass
k= Initial microorganisms count estimated by the modified Gompertz model (Log_{10} CFU/g)
A=*M*aximum microorganisms growth attained at the stationary phase (Log_{10} CFU/g)
μ_{max} = Maximal growth rate [Δ Log10 (CFU/g)/day]
λ= Lag time (days)
MSE= Mean Square Error
R^2 = Correlation coefficient

Note: Gompertz parameters for mesophilic, psychrophilic and yeast and molds growth in apple pieces coated with EC + EOs in high concentrations or their active compounds were not calculated because growth through the storage time was not observed, indicating that shelf-life of those samples was much longer than 30 days.

In general, EC including malic acid at 2.5% (w/v) resulted effective to inhibit the populations of native flora during the storage time, since longer lag times (λ) and lower growth rates (μmax) for mesophilic, psychrophilic and yeast and molds populations were observed in comparison with non-coated apple pieces (Tables 3). Those results are according with those reported by Baldwin *et al*., (*2*), Perez-Gago *et al*., (*42*)

and Olivas and Barbosa-Cánovas (*35*) who indicated that native flora can be inhibited by modified atmosphere originated by the edible coatings as well as with those reported by Raybaudi-Massilia *et al*., (*45*) who demonstrated that malic acid at 2.5% (w/v) can inhibit the growth of mesophilic and psychrophilic populations in fresh-cut apples stored at 5ºC. Nevertheless, the incorporation of EOs into the edible coating even more inhibited the proliferation of the studied populations, since, longer lag times (λ) and lower growth rates (μmax) than those observed in fresh-cut apples coated with EC were found when those substances were used (Tables 3), indicating that an enhanced antimicrobial effect was caused by the combined incorporation of malic acid and EOs. That inhibitory effect of edible coatings was particularly intensified when higher concentrations of EOs or their active compounds were added, being the populations studied not detected during 30 days of storage (Fig. 1). There are very few works in the literature that demonstrating the effects of essential oils incorporated into the edible coatings applied to fresh-cut fruits. In such sense, Rojas-Graü *et al*., (*50*) indicated that alginate-apple pure edible coatings with lemongrass (1 and 1.5% v/v) or oregano (0.3 and 0.5% v/v) oils were effectives to control both bacterial and fungi growth in fresh-cut "Fuji" apples. Otherwise, studies based in direct application of EOs or their active compounds to fresh-cut fruits have demonstrated that native microflora can be reduced or inhibited using those substances. Hence, Lanciotti *et al*., (*24*) reported that the addition of 0.02% (v/v) of citrus, mandarin, cider, lemon and lime EOs to a fresh sliced fruit mixture (apple, pear, grape, peach and kiwifruit) inhibited the proliferation of natural occurring microbial flora and reduced the growth rate of inoculated *Saccharomyces cerevisiae* population. Likewise, Roller and Seedhar (*54*) demonstrated that carvacrol and cinnamic acid were effective to reduce and inhibit the natural flora growth in fresh-cut kiwifruit and honeydew melon, respectively, when were used in a concentration of 0.015% (v/v).

Microbiological shelf-life. Shelf-life of non-coated fresh-cut apples was mainly limited by mesophilic microorganisms, whereas shelf-life of coated fresh-cut apples was limited by fungi (Table 3). Similar results were reported by Raybaudi-Massilia *et al*., (*45*) who indicated that mesophilic flora limited the shelf-life of fresh-cut apples stored at 5ºC. Antibacterial action of malic acid as well as the modified atmosphere (high carbon dioxide and low oxygen relative) created by the edible coating could be the causes of the change of predominant flora observed in those coated apples pieces, since a higher inhibition of mesophilic aerobic flora and an

increasing in the fungi proliferation, mainly yeasts which is a flora more acid tolerant and generally fermentative was observed (Table 3, Fig. 1). In such sense, Alonso and Alique, (*1*) indicated that reduced interchange of gases between fruit and their immediate surroundings due to the use of coatings can drastically reduce oxygen levels and alter the respiratory metabolism producing anaerobiosis and fermentation.

Significant differences ($p<0.05$) among the microbiological shelf-life of apple pieces non-coated, coated with EC or EC + EOs or their active compounds were found (Table 3). A longer shelf-life was reached in fresh-cut apples coated with EC in comparison with those non-coated apple pieces. However, a greater extension of the shelf-life was found when EOs or their active compounds were incorporated into the edible coating (Table 3) as consequence of a significant inhibition of spoilage flora growth by the combined incorporation of malic acid and EOs into the coating. In general, the three EOs applied were effective to extend the fresh-cut apples shelf-life. Nevertheless, higher concentrations of those oils were more effective than lower concentrations. On the other hand, the active compounds incorporation also extended the shelf-life of fresh-cut apples for more than 30 days (Table 3).

Changes in headspace gas composition. Oxygen consumption and carbon dioxide production in packed fresh-cut apples with or without EC and EC + EOs or their active compounds are showed in Fig. 2a and 2b. Significant differences ($p < 0.05$) in oxygen consumption and carbon dioxide production between coated and non-coated fresh-cut apples were found. Lower oxygen consumption and carbon dioxide production in fresh-cut apples coated with EC or EC + EOs than those apple pieces non-coated was detected (Fig. 2a and 2b). Alginate-based edible coating with malic acid (EC) avoided a decrease of oxygen concentration and an increase of carbon dioxide production in headspace of apple pieces trays in comparison with fresh-cut apples non-coated (Fig. 2a and 2b), as result of a lower oxygen consumption by microbial populations, which were slightly inhibited by malic acid and modified atmosphere created by the edible coating as well as by a lower fruit tissue respiration. However, the incorporation of EOs into edible coating resulted even more effective to avoid those important changes in oxygen and carbon dioxide, being intensify its effect when higher concentrations of EOs or their active compounds were used (Fig. 2a and 2b).

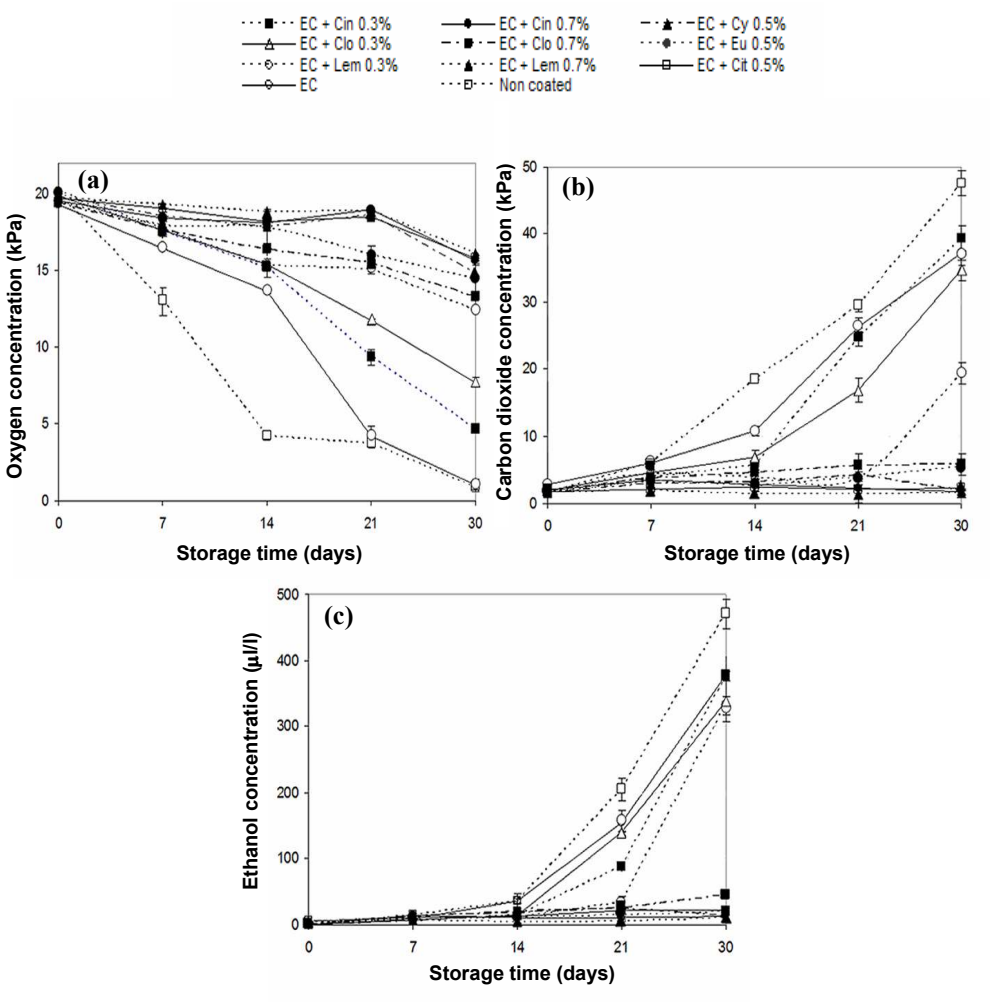

Figure 2.- Changes in oxygen (a), carbon dioxide (b) and ethanol (c) concentration in fresh-cut apples coated with alginate-based edible coating (EC) including or not EOs of cinnamon (Cin), clove (Clo) and Lemongrass (Lem) at 0.3 and 0.7% (v/v) or their active compounds: cinnamaldehyde (Cy), eugenol (Eu) and citral (Cit) at 0.5% (v/v). The values represented are mean of 4 determinations ± standard deviation.

A drastic inhibition of native microflora growth caused by a combined incorporation of malic acid and EOs in high concentrations or their active compounds into the coating can mainly explain that lower consumption of oxygen and production of carbon dioxide in those apple pieces coated with EC + EOs, whereas in non-coated apple pieces the high oxygen consumption and carbon dioxide production observed can be explained by the respiration of naturally occurring microbial flora which significantly increased through the storage time in those samples as well as by fruit tissue respiration as was previously reported by others authors (*45, 49, 55, 56, 58*).

Important reductions of oxygen consumption and carbon dioxide production in fresh-cut apples coated with apple-puree alginate edible coating with EOs incorporated were also reported by Rojas-Graü et al., (*50*), indicating that O_2 and CO_2 concentrations in apple pieces coated with edible coating containing lemongrass oil (1 and 1.5% v/v) and oregano (0.5% v/v) were practically unaffected during 21 of storage.

Ethanol is an indicator of the degree of anaerobic fermentation caused by microbial growth and its accumulation can takes place when internal atmosphere is affected and gas exchange is restricted, as occur when edible coatings are used (*41*). Headspace trays of apple pieces coated with EC or EC + EOs showed lower concentrations of ethanol than those trays with apple pieces non-coated, being intensified the effect when EOs in high concentrations or their active compounds were incorporated into the coating (Fig. 2c). A higher inhibition of native flora growth caused by the combined incorporation of malic acid + EOs into the coating (Fig. 1), which implicated a lower grade of fermentation in apple pieces coated with EC + EOs in high concentrations or their active compounds can explain the lower ethanol production detected in those samples in comparison with those apple pieces coated with EC or non-coated where native flora increase more significantly.

Firmness changes. Notables differences in apple pieces firmness dependents on treatment condition were not found at t = 0 day, with the exception of those apple pieces coated with EC + citral (0.5% v/v) where a important reduction of this parameter was observed at that time, whereas a significant influence of EOs over firmness samples through the storage time was detected (Table 4).

Table 4.- Firmness of fresh-cut apples coated with EC including or not EOs or their active compounds stored at 5ºC for 30 days

Treatment	Firmness [α] Storage time (days)					Shelf-life [δ] (days)
	0	7	14	21	30	
Non-coated	10.6 ± 0.9 Aa	9.5 ± 1.2 Ab	9.0 ± 0.5 Ac	7.51 ± 0.25 ABd	5.8 ± 0.7 Ae	>21
EC	10.03 ± 0.05 ABa	10.1 ± 0.7 Aa	9.62 ± 0.24 ABa	8.30 ± 0.29 Ab	8.1 ± 0.8 Cb	>30
EC + Cin (0.3%)	9.5 ± 0.3 Ba	9.76 ± 0.16 Aa	9.87 ± 0.26 Ba	9.63 ± 0.22 Fa	8.06 ± 0.13 Cb	>30
EC + Cin (0.7%)	9.74 ± 0.04 Ba	8.22 ± 0.22 CDb	8.1 ± 0.6 Cb	[β] 6.1 ± 0.6 CDc	5.949 ± 0.027 ABc	>14
EC + Cy (0.5%)	9.95 ± 0.13 ABa	9.3 ± 0.3 ABCb	7.6 ± 0.8 Cc	6.4 ± 0.3 Cd	6.01 ± 0.11 ABd	>14
EC + Clo (0.3%)	9.7 ± 0.4 Ba	9.5 ± 1.2 ABa	9.75 ± 0.09 ABa	9.60 ± 0.15 Fa	8.21 ± 0.09 Ca	>30
EC + Clo (0.7%)	9.9 ± 0.5 ABa	9.8 ± 0.5 Aa	9.79 ± 0.11 ABa	7.9 ± 0.8 Ab	6.0 ± 1.0 ABb	>21
EC + Eu (0.5%)	9.8 ± 0.4 Ba	9.14 ± 0.09 ABCa	7.39 ± 0.29 CDb	6.5 ± 0.6 Cbc	5.95 ± 0.06 ABc	>14
EC + Lem (0.3%)	9.81 ± 0.14 Ba	9.41 ± 0.15 ABa	9.16 ± 0.26 ABa	7.5 ± 0.6 ABb	6.79 ± 0.16 Bb	>30
EC + Lem (0.7%)	9.4 ± 0.4 Ba	8.3 ± 0.6 BCDb	6.32 ± 0.04 Dc	5.28 ± 0.10 DEd	4.72 ± 0.28 Dd	>14
EC + Cit (0.5%)	8.5 ± 0.3 Ca	7.9 ± 0.6 Da	5.8 ± 0.4 Eb	4.6 ± 0.4 Ec	3.6 ± 0.4 Ec	>7

[α] Results are the mean of 10 determinations ± standard deviation

[β] Reference point below which was sensorially undesirable

[δ] Shelf-life based on the reference point

EC = Edible coating; Cin = Cinnamon; Cy = Cinnamaldehyde; Clo = Clove; Eu = Eugenol; Lem = Lemongrass; Cit = Citral

Values in the same column that are not followed by the same capital letter are significantly different ($p < 0.05$); values in the same row that are not followed by the same lower-case letter are significantly different ($p < 0.05$).

Firmness loss of fresh-cut fruits occur normally as a consequence of releasing of calcium, potassium and some enzymes pectic from fruit by cellular damages caused during its processing (*22*). Fresh-cut apples coated with EC without EOs maintained higher values of firmness through the storage time than non-coated fresh-cut apples (Table 4) due to the incorporation of calcium into edible coating which strengthened the molecular bonding between constituents of cell wall avoiding the firmness loss of apple pieces by more time. This behavior have also been reported by Olivas and Barbosa-Cánovas (*35*) and Rojas-Graü *et al*., (*50, 52*), but using calcium chloride into edible coating. However, that effectiveness of calcium lactate incorporated into the coating was reduced when EOs in high concentrations or their active compounds were added, showing those apple pieces coated with EC + EOs of lemongrass or cinnamon at 0.7% v/v or their active compounds citral and cinnamaldehyde at 0.5% v/v higher decreases.

Although there are not studies reported in the literature about the action of EOs on the cell wall of vegetal tissue, we considered that significant damages caused by those EOs and their active compounds on the fresh-cut apple tissue through the storage time could induce the releasing of some constituents of importance vital for maintaining of the cell wall integrity, thus generating, important reductions in the firmness of those samples. Similar results were reported by Rojas-Graü et al., (*50*) who indicated that apple pieces coated with apple puree-alginate edible coating containing 1.5% v/v of lemongrass oil or 0.5% v/v of oregano oil showed a drastic reduction in firmness.

In this study, end of the shelf-life of apple pieces was considered from a firmness value ≤ 6.1 N (Table 4) due to its soft aspect, which was sensorially undesirable. In such sense, coated fresh-cut apples with EC exhibited a greater shelf-life (> 30 days) than non-coated fresh-cut apples (>21 days). On the other hand, shelf-life of apple pieces coated with EC + EOs or their active compounds significantly varied (Table 4), showing apple pieces coated with EC + citral shortest shelf-life.

Color changes. Significant differences ($p < 0.05$) in the lightness of apples pieces non-coated or coated with EC or EC + EOs or active compounds at t = 0 days were not found. However, meaningful differences ($P < 0.05$) among treatment after 14 days of storage at 5ºC were observed (Table 5). In addition, significant differences ($p < 0.05$) through the storage time for each treatment condition were detected. Visible degradation of the lightness in apple pieces was considered at 67.2 L* value as is indicated in Table 5, thus, values of L* equal or below of that value were considered sensorially undesirable. Lightness was affected in higher grade in apple pieces coated with EC containing lemongrass (0.3 or 0.7% v/v), clove (0.7% v/v) and eugenol (0.5% v/v) in comparison with the rest of the treatment including those apple pieces non-coated (Table 5). Decreases in lightness of fresh-cut apples through the storage time have also been reported by Soliva-Fortuny *et al*. (*57, 58*) and Rojas-Graü *et al*. (*49*) in "Golden delicious" and "Fuji" fresh-cut apples, respectively.

On the other hand, significant differences ($p< 0.05$) among hue angle (hº) values of fresh-cut apples non-coated and coated with EC or EC + EOs or active compounds were found (Table 5). Cinnamon and lemongrass oil at 0.7% (v/v) and their active compounds cinnamaldehyde citral at 0.5% (v/v) affected more rapidly the color (hº) than others substances; whereas, non-coated fresh-cut apples exhibited the lowest hº values from the sample preparation day (t = 0 days).

Table 5.- Changes in color parameters of fresh-cut apples coated with EC including or not EOs or their active compounds and stored at 5ºC for 30 days.

Parameter [α]	Treatment	Storage time (days)					Shelf-life [δ] (days)
		0	7	14	21	30	
Lightness (L*)	**Non-coated**	73.43 ± 0.19 Aa	73.502 ± 0.014 Aa	72.45 ± 0.19 Ab	68.1 ± 0.3ABc	66.9 ± 0.3 ABd	>21
	EC	74.16 ± 0.01 Aa	74.42 ± 0.12 Aa	73.2 ± 1.5 Aab	72.5 ± 0.9 Dab	71.3 ± 0.7 Db	>30
	EC + Cin (0.3%)	74.17 ± 0.06 Aa	73.0 ± 0.4 Aab	72.6 ± 0.9 Ab	70.7 ± 0.5 ADc	70.6 ± 0.6 CDc	>30
	EC + Cin (0.7%)	74.0 ± 0.4 Aa	73.4 ± 1.8 Aab	72.3 ± 0.5 Aabc	71.5 ± 0.1 ADbc	70.7 ± 0.3 CDc	>30
	EC + Cy (0.5%)	74.73 ± 0.22 Aa	73.86 ± 0.15 Aa	73.77 ± 1.18 Aa	70.3 ± 0.2 ADb	67.9 ± 1.5 ABb	>30
	EC + Clo (0.3%)	74.24 ± 1.06 Aa	73.127 ± 0.016 Aa	72.0 ± 0.7 Aab	70.5 ± 1.3 ADb	69.83 ± 0.9 ACb	>30
	EC + Clo (0.7%)	73.9 ± 0.7 Aa	72.2 ± 1.4 Aa	70.1 ± 1.3 Aa	68.8 ± 2.1 BCa	67.1 ± 1.9 ABa	>14
	EC + Eu (0.5%)	74.0 ± 0.4 Aa	71.4 ± 0.3 Aab	70.6 ± 0.4 Abc	69.44 ± 0.03 BCbc	66.9 ± 2.1 ABc	>14
	EC + Lem (0.3%)	73.3 ± 0.9 Aa	71.6 ± 1.0 Aab	70.7 ± 0.4 Aab	70.1 ± 0.8 ABCb	67.2 ± 1.9 ABb	>14
	EC + Lem (0.7%)	73.2 ± 0.8 Aa	71.5 ± 0.7 Aab	70.6 ± 0.5 Aab	[β] 67.2 ± 1.3 Cbc	66.4 ± 0.2 ABc	>14
	EC + Cit (0.5%)	74.4 ± 0.8 Aa	73.93 ± 0.20 Aa	71.9 ± 0.6 Ab	71.1 ± 0.9 ABb	65.8 ± 0.8 Bc	>21
Hue angle (h°)	**Non-coated**	91.7 ± 0.9 Aa	91.3 ± 0.8 Aa	91.2 ± 0.4 Aa	90.67 ± 0.10 Aa	90.5 ± 0.3 Aa	<1
	EC	99.6 ± 0.6 Ba	99.9 ± 0.5 Ba	99.4 ± 0.2 Ba	99.6 ± 1.2 Ba	97.8 ± 0.4 Ba	>30
	EC + Cin (0.3%)	98.9 ± 0.2 BCa	98.0 ± 0.1 Ba	97.8 ± 0.2 Ca	98.8 ± 0.1 BCa	97.8 ± 0.1 Ca	>30
	EC + Cin (0.7%)	98.6 ± 0.8 BCa	98.5 ± 0.3 Ba	98.8 ± 0.3 Ca	97.77 ± 0.12 Da	97.4 ± 0.3 Ca	>14
	EC + Cy (0.5%)	98.2 ± 0.2 BCab	99.3 ± 0.6 Bb	97.8 ± 1.4 Cab	[β] 97.4 ± 0.8 Dab	95.7 ± 0.8 Ca	>14
	EC + Clo (0.3%)	99.1 ± 0.2 Ba	99.6 ± 0.4 Bb	98.11 ± 0.01 BCc	98.8 ± 1.0 BCac	98.3 ± 0.4 BCac	>30
	EC + Clo (0.7%)	98.8 ± 0.5 BCa	99.7 ± 0.4 Bb	99.68 ± 0.04 Bb	99.92 ± 0.20 Bb	98.6 ± 0.5 Ba	>30
	EC + Eu (0.5%)	98.2 ± 0.8 Ca	98.8 ± 1.5 Ba	99.2 ± 0.9 Ca	98.3 ± 0.7 Ca	98.09 ± 0.15 Ca	>30
	EC + Lem (0.3%)	98.2 ± 0.6 BCa	98.2 ± 1.4 Ba	99.1 ± 1.2 BCa	98.65 ± 0.01 Ba	97.6 ± 0.1 BCa	>30
	EC + Lem (0.7%)	98.3 ± 0.7 BCab	99.5 ± 0.7 Bb	98.04 ± 0.20 Cab	95.91 ± 0.06 Ea	95.80 ± 0.16 Ca	>14
	EC + Cit (0.5%)	98.0 ± 1.9 BCa	99.4 ± 0.2 Ba	98.4 ± 1.2 Ca	98.01 ± 0.22 CDa	97.4 ± 0.6 Ca	>14

[α] Data are the mean of 30 determinations ± standard deviation
[β] Reference point below which was sensorially undesirable
[δ] Shelf-life based on the reference point
EC = Edible coating; Cin = Cinnamon; Cy = Cinnamaldehyde; Clo = Clove; Eu = Eugenol; Lem = Lemongrass; Cit = Citral.
Values in the same column that are not followed by the same capital letter are significantly different ($p < 0.05$); values in the same row that are not followed by the same lower-case letter are significantly different ($p < 0.05$).

The latter fact is mainly due to the action of enzymes such as polyphenol oxidase (PPO) and peroxidase (POD) on cut or damaged surface of fruits, which catalyze the oxidation of phenolic compounds such as caffeoyl, coumaroyl and feruloyl (hydroxycinnamic acid derivatives mainly presents in apples) to produce brown pigments in oxygen presence (*25*). Being, this

browning greatly avoided in coated apple pieces, as a consequence of the antibrowning agents incorporated (N-acetyl-L-cysteine and glutathione) into the coating, which reacting competitively with PPO and POD by the intermediate quinones to form stable colorless compounds (*46, 47*). This effect has also been reported in fresh-cut apples by Molnar-Perl and Friedman, (*32*); Rojas-Graü *et al*., (*51*) and Raybaudi-Massilia *et al*., (*45*). Nonetheless, that browning prevention was notably affected by the incorporation of EOs in high concentrations or their active compounds into the coating, possibly as a consequence of the releasing of enzymes and substrates induced by significant damages caused by those compounds on the cell tissue integrity.

In general, EC and EC+ EOs in low concentrations extended the shelf-life of fresh-cut apples by more than 30 days with exception of lemongrass 0.3% (v/v) which affected the color in higher grade, whereas EC + EOs in high concentrations or their active compounds significantly limited the shelf-life of apple pieces (Table 5).

Sensory evaluation. Non-coated fresh-cut apples showed the lower score for color at 0 and 15 days in comparison with coated with EC or EC + EOs apple pieces, whereas significant differences in color among apple pieces coated with EC and those apple pieces coated with EC + EOs were not detected by the panelists (Fig. 3). Apple pieces coated with EC were the preferred by the panelists at t = 0 days, showing the highest scores for color, firmness, taste and acidity, indicating that EC did not modify the sensory attributes of the fresh-cut apples. That result is in according to that reported by Debeaufort *et al*., *(10)* and Rojas-Graü *et al*., (*50*) who indicated that edible coatings can avoid partial or total loss of food flavors, thus maintaining their quality. However, the incorporation of EOs into the coating significantly influenced over the odor and taste characteristics of apple pieces at t = 0 day (Fig.3), showing the apple pieces coated with EC + clove the lower score in taste. On the other hand, significant differences ($p < 0.05$) among odor, acidity and taste of apple pieces coated with EC and EC + different EOs were found after 15 days of storage, being those fresh-cut apples coated with EC and EC + lemongrass the preferred by the panelist.

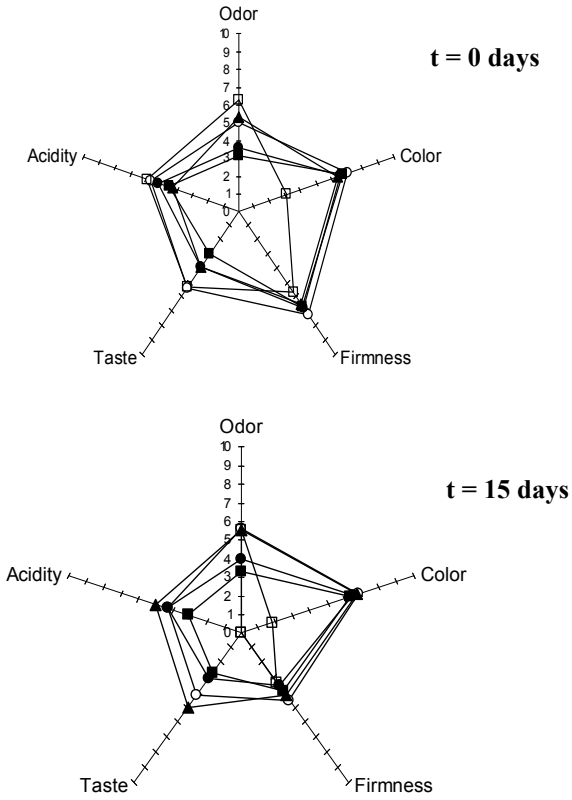

Figure 3.- Influence of storage time on sensory characteristic of fresh-cut apples coated with alginate-based edible coating including EOs of cinnamon (●), clove (■) and Lemongrass (▲) at 0.7% (v/v). Controls with edible coating without essential oils (○) and fresh-cut apples non-coated (□) also were evaluated. The values are mean of 30 determinations. Taste and acidity determination at 15 days in fresh-cut apples non-coated were not carried out due to its high microbiological load.

CONCLUSIONS

Alginate-based edible coating containing malic acid and stabilizing substances reduced the inoculated *E. coli* O157:H7 population in fresh-cut apples and also inhibited the growth of native microflora in comparison with non-coated fresh-cut apples. However, the incorporation of EOs or their actives compounds into EC resulted more effective in reducing those pathogenic and spoilage populations. More than 4 Log CFU/g reductions of *E. coli* O157:H7 and a total inhibition of native flora was achieved in apple pieces coated with EC + EOs in high concentrations or their active compounds stored at 5ºC for 30 days.

Among the selected EOs and active compounds, lemongrass and cinnamon oil (0.7% v/v), cynnamaldehyde (0.5% v/v) and citral (0.5% v/v) were the most effective from a microbiological point of view, whereas, EOs of lemongrass, cinnamon and clove in low concentrations better maintained the physicochemical characteristics of the apple pieces. On the other hand, apple pieces coated with EC were preferred by the panelists at t = 0 days, whereas, apple pieces coated with EC and EC + lemongrass at 0.7% (v/v) were sensorially preferred by the panelists after 15 days of storage.

According to the results EC acted as a good anti-browning, anti-softening and anti-microbial substances carrier, since an extension of the fresh-cut apples shelf-life from physicochemical (> 30 days) and microbiological (19 days) point of view was accomplished in comparison with non-coated apple pieces. However, the incorporation of EOs in high concentrations or their active compounds into the EC affected physicochemical characteristics of apple pieces, limiting their shelf-life between 7 and 21 days.

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Edible alginate-based coating as carrier of antimicrobials to improve shelf-life and safety of fresh-cut melon

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ABSTRACT

The effect of malic acid and essential oils (EOs) of cinnamon, palmarosa and lemongrass and their main active compounds as natural antimicrobial substances incorporated into an alginate-based edible coating on the shelf-life and safety of fresh-cut "Piel de Sapo" melon (*Cucumis melo* L.) was investigated. Melon pieces (50g) were coated with alginate-based edible coating containing malic acid (EC) and EOs or their active compounds before to be packed in air filled polypropylene trays and stored at 5ºC for shelf-life and sensory studies. On the other hand, melon pieces were inoculated with a *Salmonella* Enteritidis (10^8 CFU/ml) culture before applying the coatings containing malic acid and EOs or their active compounds to safety study. Controls of fresh-cut melon non-coated or coated with EC without EOs were also prepared. EC was effective to improve shelf-life of fresh-cut melon from microbiological (up to 9.6 days) and physicochemical (>14 days) points of view in comparison with non-coated fresh-cut melon, where microbiological and physicochemical shelf-life was up to 3.6 days and lower than 14 days, respectively. In addition, the incorporation of EOs or their active compounds into the edible coating prolonged the microbiological shelf-life by more than 21 days in some cases due probably to an enhanced antimicrobial effect of malic acid + EOs; however, some fresh-cut melon characteristics were affected such as firmness and color causing a reduction of physicochemical shelf-life. Significant reductions ($p<0.05$) of *S*. Enteritidis population in inoculated coated fresh-cut melon were achieved, varying the effectiveness of the coatings depending on the EOs or the active compound and their concentrations. According to the results, palmarosa oil incorporated at 0.3% into the coating appear to be a promising preservation alternative for fresh-cut melon, since it had a good acceptation by panellists, maintained the fruit quality parameters, inhibited the native flora growth and reduced *S*. Enteritidis population.

Key words: Edible coating, alginate, melon, *S*. Enteritidis, malic acid, essential oils, shelf-life

1-INTRODUCTION

Ready to eat fresh fruit has become an important area of potential growth in the fast expanding produce industry (Corbo et al., 2000), presumably due, in part, to their characteristics of freshness, low caloric contents, commodity to be used and an active promotion of fruits and vegetables as basic components of a healthy diet. Nevertheless, it is well known that minimal processing alters the integrity of the fruit and induce surface damages increasing lightly the tissue respiration and leading biochemical deteriorations such as browning, off-flavour development and texture breakdown decreasing the fresh-cut fruit quality (Lee et al., 2003; Martín-Belloso et al., 2006, Rojas-Graü et al., 2006a; Oms-Oliu et al., 2007; Raybaudi-Massilia et al., 2007). In addition, contamination of the fruit flesh can occur from the skim, increasing the fruit spoilage and the risk to consumer due to pathogenic microorganisms presence.

Different serovars of *Salmonella* as well as *E. coli* O157:H7 have been implicated in foodborne outbreaks by consumption of Cantaloupe, Honeydew and watermelon, occurred from 1950 to 2003 (Harris et al., 2003; Bowen, et al., 2006; CDC, 2007). The majority outbreaks were associated with the consumption of those fruits from salad bars or fruit salads. The association of illnesses with cut-melon suggests that salmonellae may have been introduced into the fruit from the rind by the physical act of cutting the melon, or contact by cut pieces of melon with contaminated rinds (Golden et al., 1993).

Edible coatings are used commercially to reduce moisture loss, prevent physical damage, enhance product appearance and carry food ingredients including antibrowning agents, colorants, flavours, nutrients, spices and antimicrobials (Martín-Belloso et al., 2005; Franssen and Krochta, 2003). The functionality of edible coatings can be expanded by incorporating antimicrobials to protect food products from microbial spoilage, extend their shelf-life and enhance their safety (Franssen and Krochta, 2003; Cagri et al., 2004; Oussalah et al., 2006; Rojas-Graü et al., 2007a). Therefore, the use of edible coatings in a wide range of food products, including fresh and minimally processed fruits, is receiving great interest by food industry.

The effectiveness against several pathogens of different antimicrobial substances such as lysozyme, nisin, organics acids, essential oils and their derivatives incorporated into the edible films have showed to be satisfactory (Padgett, et al., 1998; Eswaranandam et al., 2004; Cagri et al., 2004; Pranoto et al., 2005; Oussalah et al., 2006; Rojas-Graü et al.,

2006b). However, very few studies about the effectiveness of the incorporation of those compounds into edible coatings applied to fruit for ensuring quality and safety have been published (Eswaranandam et al., 2006; Rojas-Graü et al., 2007a).

Malic acid and other organic acids as well as EOs are considered as Generally Recognized as Safe (GRAS) according to Food Additive Status List (FDA, 2006) and have been used in the food industry as preservative, antioxidant and flavoring agents (Stratford and Eklund 2003; Burt, 2004). On the other hand, the effectiveness of those compounds to extend the shelf-life and control the pathogenic microorganisms growth have been demonstrated in fruit juice and fresh-cut fruit (Friedman et al., 2004; Raybaudi-Massilia et al., 2006; Rupasinghe et al., 2006; Rojas-Graü et al., 2007a). Therefore, they represent a good alternative as chemical preservatives to be included in edibles coatings to extend shelf-life of fresh-cut fruit and enhance their safety.

The objective of this study was to evaluate the effectiveness of malic acid and essential oils incorporated into alginate-based edible coating to improve the shelf-life and safety of fresh-cut melon "Piel de Sapo".

2- MATERIALS AND METHODS

2.1-Materials and sample preparation

2.1.1- Melons

*"*Piel de Sapo*"* melons **(*Cucumis melo* L.)** at commercial ripeness were provided by the Polytechnic University of Valencia (Valencia, Spain) and maintained at 10ºC until processing. A flesh characterization was carried out following the official methods of fruit juices and other vegetables and derivatives (B.O.E., 1988). Total acidity, expressed as g of citric acid /100 ml, pH (Crison 2001 pH-meter; Crison Instruments S. A., Barcelona, Spain), soluble solids content (%) (Atago RX-1000 refractometer; Atago Company Ltd, Japan), firmness (TA-TX2 Texture Analyzer, Stable Micro Systems LTD, Surrey, England) and color (Tri-stimulus Minolta CR-400 Chroma Meter, Konica Minolta Sensing, INC, Osaka, Japan) were measured.

2.1.2- Edible coating Solutions

Edible coating solutions were elaborated from a base solution of alginate (Food grade sodium alginate, Keltone LV, ISP, San Diego, CA.,

USA) at 2% w/v and glycerol (Merck, Whitehouse station, N.J., USA) at 1.5% v/v which were prepared into sterile distilled water heated previously at 70ºC and stirred until total dissolution of the components. Essential oils (EOs) such as Cinnamon leaf (*Cinnamomum zeylanicum*), palmarosa (*Cymbopogon martini*) and lemongrass (*Cymbopogon citrates*) were added at 0.3 and 0.7%; whereas, their active compounds such as eugenol, geraniol and citral were incorporated into the coating base solution at 0.5%. The EOs and their compounds were purchased by Aceites Esenciales Dicana (Barcelona, Spain). Edible coating without essential oil (EC), as control, was also evaluated. Those solutions were mixed for 3 min using an Ultra Turrax T25 homogenizer (IKA® WERKE, Germany) with a S25N-G25G device, and then degassed through a vacuum pump (Vaccubrand, Wertheim, Germany). In addition, a bath with aqueous solution containing calcium lactate pent-hydrate (Scharlau Chemie S. A. Barcelona, Spain) at 2% w/v and D-L malic acid (Scharlau Chemie S. A.) at 2.5% into sterile distilled water was used to crosslink the carbohydrate polymers. The concentrations selected for this work were based in previous researches (Raybaudi-Massilia et al., 2006; Raybaudi-Massilia et al., 2007; Rojas-Graü et al., 2007a; 2007b).

2.1.3.- Strain and inoculum preparation

A strain of *Salmonella enterica* serovar Enteritidis 1.82 (NCTC 9001) from the National Collection of Type Culture (PHLS, Central Public Health) was utilized in this research. The strain was maintained in Tryptone soy agar (TSA) (Biokar Diagnostics. Beauvais, France) slants at 5ºC. Stock culture of *S.* Enteritidis was grown in tryptone soy broth (TSB) at 37ºC for 11 hours and 120 rpm. The maximum level reached for the microorganism was 4.8 x 10^9 colony forming units/ millilitre (CFU/ml). Concentration was then adjusted to 10^8 (CFU/ml) using saline peptone water (0.1% peptone, Biokar Diagnostics., plus 0.85% NaCl, Scharlau Chemie, S.A.).

2.1.4- Melon processing and packaging

"Piel de Sapo" melons were sanitized by immersion in sodium hypochlorite solution (300 ppm); then washed with potable water and finally dried with absorbent paper. The cleaned fruit was cut in slices of 3 cm width with a knife and then cut with a cylindrical hollow instrument of stainless steel of sharpened brink in pieces of 1.40 cm diameter x 2.00 cm of height taken out from the fruit flesh (approximately 15-20 cylinders per melon slice). Melon pieces at room temperature (18ºC) were dipped into coating solutions for 2 min at 18ºC, afterwards residual solution was

allowed to drip off for 1 min, just before submerging the melon pieces for additional 2 min in the solution of calcium lactate with D-L malic acid at 18ºC to favours the crosslink. Ten melon pieces coated with a total weight of approximately 50 g were placed into the polypropylene trays of 173 x 129 x 35 mm. The trays were thus sealed using a thermo-sealing machine, ILPRA Food Pack Basic V/G (Ilpra , Vigenovo, Italy). The permeability of the plastic used for sealing was the following: 142.86 $fmol.s^{-1}.m^{-2}.kPa^{-1}$ to water vapour at 38ºC, 90% H.R., 52.38 $fmol.s^{-1}.m^{-2}.kPa^{-1}$ to O_2 at 23ºC, 0% H.R. and 2.38 $fmol.s^{-1}.m^{-2}.kPa^{-1}$ to CO_2 at 23ºC, 0% H.R. Finally the trays containing coated melon pieces and controls were stored at 5ºC for 21 days to be analyzed for shelf-life and sensory studies. On the other hand, ten melon pieces with a total weight of 50 g were placed into the polypropylene trays and then inoculated uniformly by spreading 500 µl of *S* Enteritidis stock cultures (10^8 CFU/ml) over its entire upper surface with a sterile micropipette for challenge study. After thirty minutes for fruit inoculum absorption the same process of coating above described was carried out for these samples. Trays were thus sealed and stored at 5ºC for 21 days. A total of 20 trays of coated non-inoculated melon pieces by each tested antimicrobial were prepared for shelf-life estimation (10 trays for physicochemical analysis and 10 trays for microbiological analysis) in order to be analyzed in duplicated at 0, 3, 7, 14 and 21 days of storage, whereas 12 trays of coated non-inoculated melon pieces were prepared by each tested antimicrobial to carry out a sensory evaluation at 0 and 15 days of storage. In addition, a total of 10 trays of coated inoculated melon pieces were prepared by each tested antimicrobial for *Salmonella* Enteritidis challenge study, whereas 10 trays of non-coated fresh-cut melon pieces and inoculated with *S*. Enteritidis were also prepared to be used as control.

2.2- Samples analyses

2.2.1.- Microbiological analysis

Counts of mesophilic, psychrophilic and yeasts and moulds populations in non-inoculated coated melon pieces trays were made at 0, 3, 7, 14 and 21 days in order to know the influence of the malic acid, EOs and their main active compounds added to edible coating solutions over the native flora. On the other hand, counts of *S*. Enteritidis in coated inoculated fresh-cut melon trays were also carried out at 0, 3, 7, 14 and 21 days of refrigerated storage to evaluate the effect of the different substances incorporated into the edible coating solutions over the *S*. Enteritids population. A pair of randomly chosen non-inoculated and inoculated

coated melon trays of each coating condition was analyzed in duplicated at each indicated time.

Mesophilic and Psychrophilic microorganisms counts were made in non-inoculated coated melon according to the ISO 4833:1991 guideline using Plate Count Agar (PCA) (Biokar Diagnostics) and pour plate method. The plates of psychrophilic microorganisms were incubated at 5ºC for 10-14 days, whereas those of mesophilic microorganisms were incubated at 35ºC for 48 h. On the other hand, yeasts and moulds counts were made according to the ISO 7954:1987 guideline using Chloramphenicol Glucose Agar (CGA) (Biokar Diagnostics) and spread plate method. The plates were incubated at room temperature (18ºC) for 3-5 days.

S. Enteritidis counts were carried out in coated inoculated melon pieces making an initial dilution of the sample (50 g of sample plus 450 ml of saline peptone water), in a masticator (IUL Instruments, Barcelona, Spain) for 1 min and preparing seriated dilutions, which were then spread at reason of 0.1 ml in Hektoen agar plates and incubated at 37ºC for 24 h. Absence of *Salmonella* serovars in fresh-cut melon was previously demonstrated through an incidence process based on ISO 6579:2002 (2002). All the microbial counts were expressed as Log_{10} CFU/g and the reported values are the mean of four determinations ± standard deviation.

2.2.2- Microbial modelling

Mesophilic, psychrophilic and yeasts and moulds growth in non-inoculated coated fresh-cut melon, expressed as Log_{10} CFU/g were modelled according to the Gompertz equation modified by Zwietering et al., (1990) (Equation 1), whereas microbiological shelf-life (***SL***) was calculated through equation (2) obtained from Gompertz modified by Zwietering et al. (1990) (Equation 1). This equation consider as maximum limit of mesophilic aerobic total count at expiry date the value allowed by the Spanish regulation for hygienic processing, distribution and commerce of prepared meals (B.O.E, 2001) which is 10^7 CFU/g.

$$Y = k + A \cdot exp\{-exp[(\mu_{max} \cdot 2.7182 / A)(\lambda - t) + 1]\} \quad (1)$$

$$SL = \lambda - \frac{A\left\{ln\left[-ln\left(\frac{log(10^7) - k}{A}\right)\right] - 1\right\}}{\mu_{max} \cdot 2.7182} \quad (2)$$

where *Y*, is the count of microorganisms (log_{10} CFU/g) for a given time; *k*, is the microorganism initial count estimated by the model (log_{10} CFU/g); *A*, is the maximum microorganism growth attained at the stationary phase (log_{10} CFU/g); μ_{max}, is the maximal growth rate [Δ log_{10} (CFU/g)/day]; λ, is the lag time (days), *t,* is the storage time (days) and *SL*, is the microbiological shelf-life time.

2.2.3- Headspace gases analysis

The gas composition of the package headspace was determined using a Micro-GP CP 2002 gas analyzer (Chrompack International, Middelburg, Netherlands) equipped with a thermal conductivity detector. A sample of 1.7 ml was automatically withdrawn from the headspace atmosphere. Portions of 0.25 and 0.33 µl were injected for O_2 and CO_2 determination, respectively. The O_2 content was analyzed with a CP-Molsieve 5Å packed column (Chrompack International) (4m x 0.32mm, df =10mm) at 60ºC and 100 kPa. On the other hand, a Pora-PLOT Q column (Chrompack International) (10m x 0.32mm, df =10mm) was held at 70ºC and 200 kPa for CO_2, ethylene, and ethanol quantification. A pair of trays of non-inoculated fresh-cut melon for each edible coating condition and the control randomly taken was analyzed in duplicated at 0, 7, 14 and 21 days of storage. Thus, the reported values are the mean of four determinations ± standard deviation.

2.2.4.- Color determination

The color of fresh-cut melons was determined in 10 pieces from a pair of trays for each edible coating condition and the control randomly chosen using a tri-stimulus Minolta CR-400 Chroma Meter (Konica Minolta Sensing, INC. Osaka, Japan) with the illumining D75 and observation angle of 10º, calibrated with a standard white plate (Y = 94.00, x = 0.3158, y = 0.3322). Three readings of L* (Lightness), a* (green chromaticity) and b* (yellow chromaticity) coordinates were recorded for each melon piece. Numerical values of L*, a* and b* parameters were employed to calculated whiteness index (WI) and chroma (C*) through the equations 3 and 4, respectively:

$$WI = 100 - \left[(100 - L^*)^2 + (a^*)^2 + (b^*)^2\right]^{1/2} \quad (3)$$

$$C^* = \left[(a^*)^2 + (b^*)^2\right]^{1/2} \quad (4)$$

The reported values are the mean of thirty determinations ± standard deviation.

2.2.5. - Firmness determination

The firmness determination was performed in ten pieces from a pair of melon pieces trays for each edible coating condition and the control, using a TA-TX2 Texture Analyzer (Stable Micro Systems LTD. Surrey, England) and the following conditions: Pre-test speed: 2 mm/s, test speed: 5.0 mm/s, post-test speed: 5.0 mm/s and penetration distance: 10 mm. The resistance of penetration was measured as the strength necessary for a cylindrical probe of 4 mm of diameter and plane basis to penetrate a cylindrical sample of pear flesh of 2.00 cm height. Five pieces of each tray were analyzed, being the reported values the mean of 10 determinations ± standard deviation.

2.2.6. – Sensory evaluation

Fresh-cut melons non-coated, coated with EC without EOs and coated with EC + EOs of cinnamon, palmarosa or lemongrass at 0.7% were used to carry out a sensory analysis in a similar way as Saftner et al., (2005). Pieces of melon prepared the same day of the test (t = 0) and also those stored at 5ºC for 15 days were evaluated by thirty volunteers aged between 20-50 years old, who like and eat melon frequently. A total of 10 samples (5 prepared at t = 0 days and 5 stored for 8 days) labelled with 3 digit code numbers were randomly provided to the panellists. The evaluated samples by each time comprised three samples with EC including EOs, one sample with EC without EOs and one sample of fresh-cut melon non-coated. A glass containing potable water and pieces of non-salted cracker were provided to panellists for eliminating the residual taste between samples. Odour, color, taste, and firmness were the evaluated attributes by the panellists. Each attribute was scored on a structured hedonic scale labelled from "extremely unpleasant" (0) to "extremely pleasant" (10).

2.3 - Statistical analysis

Statistical analysis was performed using the statistical package Statgraphics Plus version 5.1. A multifactor ANOVA with posterior Multiple Range Test was used to find significant differences ($p < 0.05$)

among storage time and edible coating condition on microbiological counts, headspace gas composition, firmness, color and sensory evaluation profile.

3- RESULTS AND DISCUSSION

3.1-Flesh fruit characterization

A physicochemical characterization of the flesh melon was carried out to offer detailed information about the fruit used in this study. A total acidity of 0.286 ± 0.003 g of citric acid /100 ml, pH 6.06 ± 0.01, soluble solids content 12.7 ± 0.8 (%), texture 4.03 ± 0.24 N and color L*= 67.91 ± 0.33, a*= -2.12 ± 0.20 and b*= 8.43 ± 0.25 were found. Similar results for total acidity (0.288 ± 0.009) and pH (5.95 ± 0.08) were reported by Oms-Oliu et al., (2007) in fresh-cut "Piel de Sapo" melon at Green mature ripeness stage (commercial ripeness). However, lower values for color (L*= 60.2 ± 0.07, a*= -1.76 ± 0.09 and b*= 5.9310 ± 0.0007) and higher for soluble solids content (13.45 ± 0.07) were found by those authors in comparison with us.

3.2-Effect of edible alginate coating containing malic acid and EOs on the native flora in fresh-cut melon

EC was effective in reducing levels of mesophilic and psychrophilic microorganisms in fresh-cut melon, since significant differences ($p<0.05$) between the counts of those populations were found between coated and non-coated fresh-cut melon after a multifactor ANOVA analysis (Fig. 1, 2 and 3). However, the incorporation of EOs or their main actives compounds into the edible coating resulted in a greater effectiveness to reduce native flora, thus observing, an enhanced antimicrobial effect mainly caused by the combination of malic acid and EOs. In addition, the effectiveness of the edible coatings varied significantly ($p<0.05$) depending on the essential oil or active compound incorporated and their concentrations (Fig. 1, 2 and 3).

3.2.1-Mesophilic aerobic microorganisms

Initial population of mesophilic microorganisms on just processed non-coated fresh-cut melon were approximately 4 Log_{10} CFU/g. Similar results were reported by Bai et al., (2001) and Oms-Oliu et al., (2006) who found initial counts of 3 Log CFU/g in "Cantaloupe" and "Piel de Sapo" melon at similar ripeness stage. In addition, significant differences ($p<0.05$) were observed when the initial populations of mesophilic microorganisms

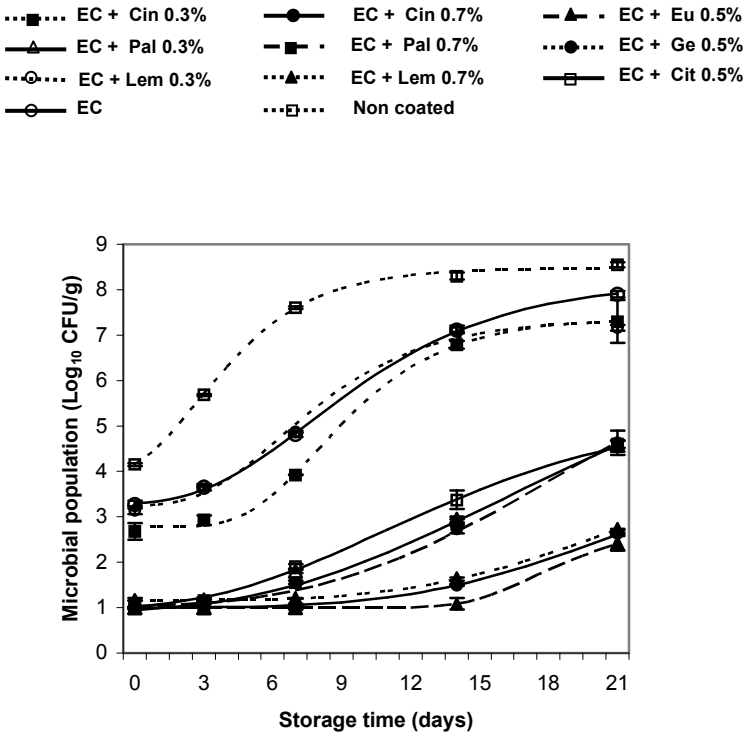


Figure 1.- Growth of mesophilic microorganisms in non-inoculated fresh-cut melon pieces with edible alginate coating (EC) including or not EOs of cinnamon (Cin), palmarosa (Pal), lemongrass (Lem) or their active compounds eugenol (Eu), geraniol (Ge) and citral (Cit). Points represented with geometrical figures indicate values observed, whereas lines represent fitted curves by Gompertz modified equation. The values represented are the mean of 4 determinations ± standard deviation. Detection limits 1 Log_{10} CFU/g.

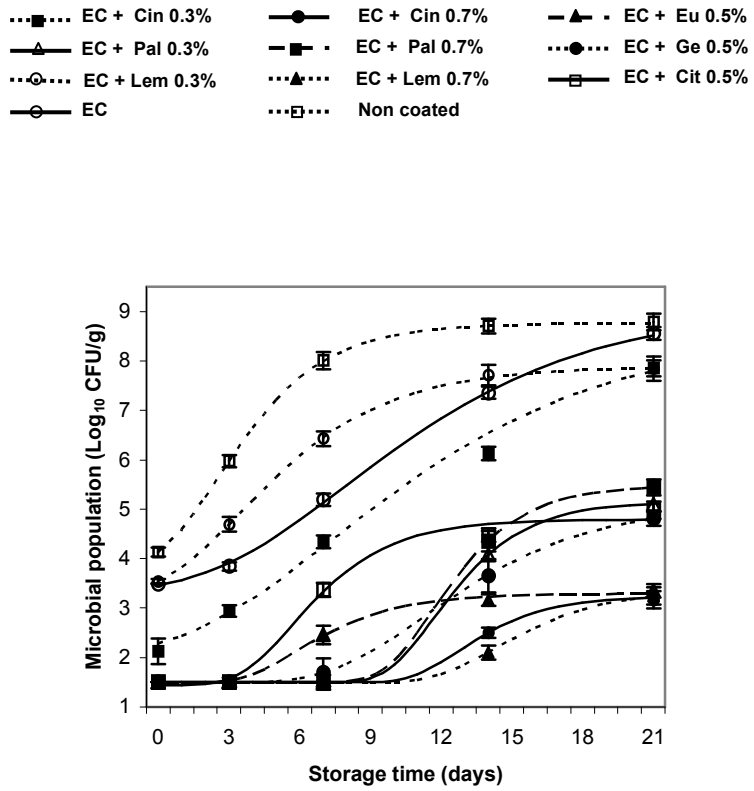


Figure 2.- Growth of Psychrophilic microorganisms in non-inoculated fresh-cut melon pieces with edible alginate coating (EC) including or not EOs of cinnamon (Cin), palmarosa (Pal), lemongrass (Lem) or their active compounds eugenol (Eu), geraniol (Ge) and citral (Cit). Points represented with geometrical figures indicate values observed, whereas lines represent fitted curves by Gompertz modified equation. The values represented are the mean of 4 determinations ± standard deviation. Detection limits 1 Log_{10} CFU/g.

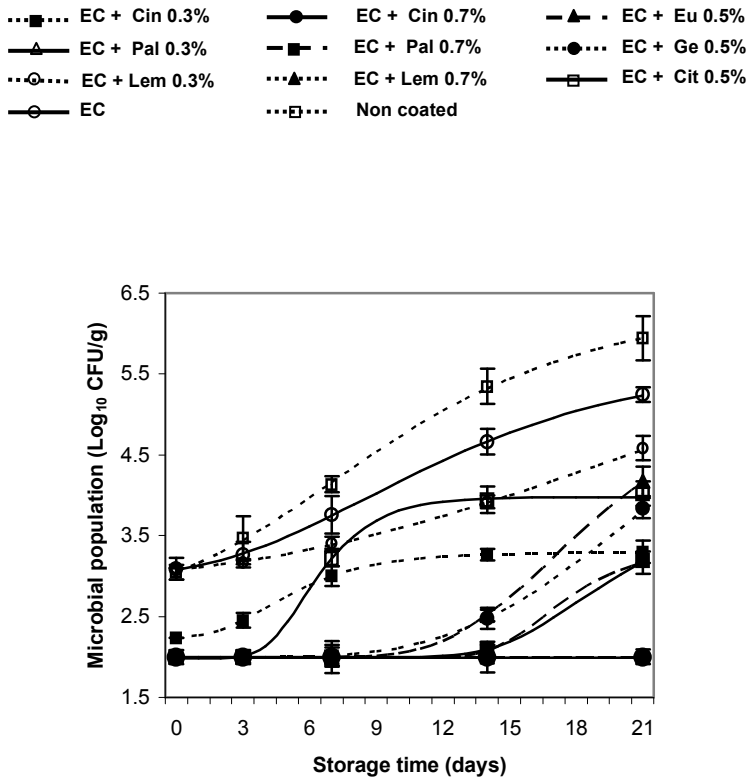


Figure 3.- Growth of yeasts and moulds in non-inoculated fresh-cut melon pieces with edible alginate coating (EC) including or not EOs of cinnamon (Cin), palmarosa (Pal), lemongrass (Lem) or their active compounds eugenol (Eu), geraniol (Ge) and citral (Cit). Points represented with geometrical figures indicate values observed, whereas lines represent fitted curves by Gompertz modified equation. The values represented are the mean of 4 determinations ± standard deviation. Detection limits 2 Log_{10} CFU/g.

were compared among fresh-cut melons with different pre-treatment: non-coated, coated with EC and coated with EC containing different EOs, being the counts found in non-coated fresh-cut melon higher than those observed in those coated fresh-cut melon (Fig. 1). The difference observed between fresh-cut melon non-coated or coated with EC could be caused by the antimicrobial activity of malic acid added to the crosslink solution, whereas the differences found among fresh-cut melon coated with EC or with EC + different EOs is consequence of the antimicrobial activity of the combination of malic acid and EOs or their active compounds and their concentrations.

Data modelling by modified Gompertz model (Eq. 1) allowed predicting in a mathematical form the aerobic mesophilic microorganisms growth in non-coated and coated fresh-cut melon during 21 days of refrigerated storage. A more rapid growth of mesophilic microorganisms was observed in non-coated fresh-cut melon in comparison with that coated, since a lag phase (λ) shorter and a growth rate (μ) higher were found, being the values of those parameters in non-coated fresh-cut melon 0.02 ± 0.42 days and 0.62 ± 0.03 Log_{10}CFU/g /day respectively, whereas for fresh-cut melon coated with EC those values were 2.93 ± 0.32 days and 0.388 ± 0.016 Log_{10}CFU/g /day. The modified atmosphere originated by the edible coating (Baldwin et al., 1995; Perez-Gago et al., 2003; Olivas & Barbosa-Cánovas, 2005) as well as the antimicrobial effect of malic acid over mesophilic flora could explain the inhibitory effect observed in fresh-cut melon coated with EC. In addition, the incorporation of EOs or their active compounds into the coating caused a higher inhibitory effect ranging the values of λ and μ observed for fresh-cut melon coated with EC containing different EOs from 2.9 ± 0.8 to 12.97 ± 1.36 days and 0.186 ± 0.029 to 0.55 ± 0.09 Log_{10}CFU/g /day depending on the EO and their concentrations (Table 1). In this way, EOs of cinnamon and lemongrass in a concentration of 0.7% were more effective than palmarosa since λ values longer and growth rate (μ) shorter were observed when those EOs were used (Table 1), indicating that mesophilic microorganisms growth was inhibit for more time. However, the incorporation of palmarosa into the edible coating caused a greater inhibition of the mesophilic microorganisms growth than the use of EC. Likewise, eugenol showed to inhibit better the mesophilic growth while λ and μ values equal to 14.4 ± 2.6 days and 0.23 ± 0.04 Log_{10}CFU/g/day, respectively, were observed. In contrast, geraniol showed λ and μ values up to 8.9 ± 0.9 days

and 0.34 ± 0.05 Log_{10}CFU/g /day, respectively; whereas, citral up to 3.48 ± 1.12 days and 0.231 ± 0.019 Log_{10}CFU/g /day, respectively.

Table 1.- Gompertz parameters that describe the growth of mesophilic microorganisms in fresh-cut melon pieces with edible alginate coating including or not EOs or their active compounds and stored at 5ºC for 21 days.

Treatment	R^2	MSE	Gompertz parameters[a]			
			k	A	μ_{max}	λ
Non-coated	99.84	0.0075	3.85 ± 0.17	4.63 ± 0.19	0.62 ± 0.03	0.02 ± 0.42
EC	99.90	0.0055	3.26 ± 0.06	4.93 ± 0.13	0.388 ± 0.016	2.93 ± 0.32
EC + Cin (0.3%)	99.16	0.054	2.78 ± 0.12	4.60 ± 0.26	0.55 ± 0.09	4.9 ± 0.6
EC + Cin (0.7%)	99.96	0.00029	0.997 ± 0.010	5.95 ± 2.16	0.20 ± 0.03	12.97 ± 1.36
EC + Eu (0.5%)	99.19	0.0042	1.000 ± 0.026	1.9 ± 0.7	0.23 ± 0.04	14.4 ± 2.6
EC + Pal (0.3%)	99.80	0.0061	0.89 ± 0.09	7.02 ± 1.42	0.247 ± 0.009	5.9 ± 0.6
EC + Pal (0.7%)	99.88	0.0039	1.01 ± 0.05	8.62 ± 2.07	0.294 ± 0.017	8.6 ± 0.6
EC + Ge (0.5%)	99.52	0.0166	0.99 ± 0.06	4.6 ± 07	0.34 ± 0.05	8.9 ± 0.9
EC + Lem (0.3%)	99.13	0.0414	3.23 ± 0.15	4.13 ± 0.25	0.44 ± 0.06	2.9 ± 0.8
EC + Lem (0.7%)	99.94	0.00035	1.157 ± 0.011	5.05 ± 1.99	0.186 ± 0.029	12.65 ± 1.32
EC + Cit (0.5%)	99.42	0.0177	0.98 ± 0.14	4.5 ± 0.6	0.231 ± 0.019	3.48 ± 1.12

[a] Data obtained from modified Gompertz model (Eq. 1)
EC = Edible coating; Cin = Cinnamon; Eu = Eugenol; Pal = Palmarosa; Ge = Geraniol;
Lem = Lemongrass; Cit = Citral
k= Initial microorganisms count estimated by the modified Gompertz model (Log_{10} CFU/g)
A=*M*aximum microorganisms growth attained at the stationary phase (Log_{10} CFU/g)
μ_{max} = Maximal growth rate [Δ Log10 (CFU/g)/day]
λ= Lag time (days)
MSE= Mean Square Error
R^2 = Correlation coefficient

3.2.2-Psychrophilic aerobic microorganisms

Psychrophilic population in fresh-cut melon was significantly ($p<0.05$) reduced at t = 0 days using edible coating, mainly when EOs or their active compounds were added into the edible coating. In addition, the antimicrobial activity varied depending on the EOs and active compound used and their concentrations. An initial psychrophilic population of 3.63 ± 0.08 Log_{10} CFU/g was observed in non-coated fresh-cut melon, whereas initials populations of 2.99 ± 0.01 Log_{10} CFU/g and from 0.99 ± 0.00 to 3.03 ± 0.04 Log_{10} CFU/g were found for fresh-cut melon coated with EC and with

EC containing different EOs, respectively (Fig. 2). Likewise, is important to indicate that psychrophilic microorganisms were not detected when actives compounds such as eugenol, geraniol and citral were incorporated into the edible coating (Fig. 2).

A prediction of the psychrophilic population growth in fresh-cut melon coated and non-coated during 21 days of refrigerated storage was achieved using the modified Gompertz model (Eq. 1). Psychrophilic microorganisms grow more quickly in non-coated than in coated fresh-cut melon since longer λ values and lower μ values were observed in the latter condition. In addition, higher inhibition of the psychrophilic microorganisms growth was found when EOs or their active compounds were added to edible coatings. Cinnamon was the more effective EO since longer λ and lower μ values were observed when this oil was used (Table 2). However, lemongrass and palmarosa also were good antimicrobial agents while lower μ values or λ more prolonged in comparison with those found in fresh-cut melon coated with EC and non-coated fresh-cut melon were observed. On the other hand, eugenol was the best active compound to inhibit the psychrophilic growth since a longer λ and lower μ values were observed with regard to geraniol and citral (Table 2).

3.2.3-Yeasts and moulds

EC without EOs was not significantly ($p<0.05$) effective to reduce the initial population of yeast and moulds in fresh-cut melon as well as for inhibit the growth through the storage time since similar initial and final populations were found in fresh-cut melon coated with EC and non-coated fresh-cut melon (Fig. 3). However, the incorporation of some EOs or active compounds into the edible coating was not only effective to reduce initial populations of mesophilic and psychrophilic microorganisms and inhibit their growth through the time, but also yeast and moulds population naturally present in fresh-cut melon (Fig. 3). Similar results were reported by Rojas-Graü et al., (2007a) who indicated that alginate-apple pure edible coatings with EOs were effectives to control bacterial and fungi growth in fresh-cut apple.

Modelling of the yeasts and moulds data using the modified Gompertz model (Eq. 1) allowed study the behaviour of fungi flora in fresh-cut melon during 21 days of storage. Significantly longer λ values ($p< 0.05$) were found when EOs were incorporated into the edible coating, indicating that the growth of the yeast and mould was inhibited. Essential oil of cinnamon at 0.7% and eugenol at 0.5% were the antimicrobials more effective to inhibit the yeast and moulds growth in coated fresh-cut melon since the

presence of those populations was not detected (Table 3). On the other hand, palmarosa also resulted to be effective in the two concentrations used since the growth of fungi flora was warned for 14 days approximately as indicated by the λ found (Table 3).

Table 2.- Gompertz parameters that describe the growth of psychrophilic microorganisms in fresh-cut melon pieces with edible alginate coating including or not EOs or their active compounds and stored at 5ºC for 21 days.

Treatment	R^2	MSE	Gompertz parameters[a]			
			k	A	μ_{max}	λ
Non-coated	99.94	0.0029	3.30 ± 0.10	4.96 ± 0.12	0.727 ± 0.022	0.02 ± 0.24
EC	99.85	0.009	2.83 ± 0.11	5.79 ± 0.26	0.36 ± 0.02	2.0 ± 0.5
EC + Cin (0.3%)	99.78	0.02	1.49 ± 0.27	6.6 ± 0.6	0.37 ± 0.03	0.94 ± 1.31
EC + Cin (0.7%)	99.74	0.002	0.999 ± 0.026	1.74 ± 0.05	0.311 ± 0.015	$10.72 \pm 2.7 \times 10^{-7}$
EC + Eu (0.5%)	99.43	0.004	0.999 ± 0.027	1.88 ± 0.09	0.267 ± 0.023	$11.75 \pm 3.58 \times 10^{-7}$
EC + Pal (0.3%)	99.99	7.05×10^{-6}	0.999 ± 0.001	3.65 ± 0.05	0.64 ± 0.21	9.68 ± 1.39
EC + Pal (0.7%)	99.99	7.8×10^{-5}	0.999 ± 0.004	3.99 ± 0.11	0.69 ± 0.40	9.56 ± 2.55
EC + Ge (0.5%)	98.84	0.035	0.99 ± 0.09	3.64 ± 0.32	0.33 ± 0.05	7.26 ± 1.06
EC + Lem (0.3%)	99.96	0.0017	2.75 ± 0.08	4.64 ± 0.09	0.49 ± 0.01	0.09 ± 0.24
EC + Lem (0.7%)	99.51	0.005	0.97 ± 0.05	1.82 ± 0.07	0.28 ± 0.05	3.54 ± 0.6
EC + Cit (0.5%)	99.04	0.03	0.94 ± 0.12	3.35 ± 0.18	0.53 ± 0.10	3.39 ± 0.83

Nomenclature is indicated in table 1

Table 3.- Gompertz parameters that describe the growth of yeasts and moulds in fresh-cut melon pieces with edible alginate coating including or not EOs or their active compounds and stored at 5ºC for 21 days.

Treatment	R^2	MSE	Gompertz parameters[a]			
			k	A	μ_{max}	λ
Non-coated	99.91	0.002	2.83 ± 0.09	3.52 ± 0.18	0.191 ± 0.006	0.03 ± 0.75
EC	99.89	0.001	2.99 ± 0.052	2.66 ± 0.13	0.140 ± 0.006	1.6 ± 0.6
EC + Cin (0.3%)	99.08	0.0028	2.23 ± 0.04	1.06±0.05	0.153±0.018	1.6 ± 0.5
EC + Cin (0.7%)	-	-	-	-	-	-
EC + Eu (0.5%)	-	-	-	-	-	-
EC + Pal (0.3%)	99.87	0.0004	1.999 ± 0.008	1.36 ± 0.21	0.21 ± 0.04	14.15 ± 9.53
EC + Pal (0.7%)	99.70	0.001	1.999 ± 0.013	1.9 ± 0.4	0.183 ± 0.007	$14.35 \pm 2.19 \times 10^{-8}$
EC + Ge (0.5%)	99.94	0.0004	1.999 ± 0.010	5.0 ± 2.4	0.22 ± 0.04	12.8 ± 1.3
EC + Lem (0.3%)	99.63	0.002	2.95 ± 0.15	4.3 ± 2.9	0.096 ± 0.014	4.2 ± 1.6
EC + Lem (0.7%)	99.99	0.00006	1.999 ± 0.004	3.4 ± 1.6	0.256 ± 0.016	12.2 ± 0.3
EC + Cit (0.5%)	99.82	0.002	1.98 ± 0.03	2.00 ± 0.04	0.410 ± 0.085	3.9 ± 0.7

Nomenclature is indicated in table 1

3.2.4- Microbiological shelf-life of fresh-cut melon

Significant differences ($p<0.05$) with respect to the shelf-life were found among non-coated melon pieces, coated with EC and coated with EC containing different EOs or their active compounds (Table 4). In addition, shelf-life was mainly dependent on mesophilic and psychrophilic growth since were the predominant flora in fresh-cut melon in comparison with yeasts and moulds. An extension of the shelf-life of coated fresh-cut melon (9-10 days) regarding to that non-coated fresh-cut melon (3.6 days) was reached when EC was used. However, a more prolonged shelf-life was achieved when EOs or their active compounds were incorporated into the EC as a consequence of a higher reduction of initial populations and inhibition of native flora growth mainly caused by the combination of malic acid and EOs (Table 4). Those results demonstrated that EC was good antimicrobials carrier to the fruit.

Table 4.- Estimated microbiological shelf-life of fresh-cut melon pieces with edible alginate coating including or not EOs or their active compounds and stored at 5ºC for 21 days

Treatment	Shelf-life[a] (days)		
	Mesophilic	Psychrophilic	Yeast and mould
Non-coated	3.6	3.61	> 21
EC	9.89	9.61	> 21
EC + Cin (0.3%)	13.11	13.17	> 21
EC + Cin (0.7%)	> 21	> 21	> 21
EC + Eu (0.5%)	> 21	> 21	> 21
EC + Pal (0.3%)	> 21	> 21	> 21
EC + Pal (0.7%)	> 21	> 21	> 21
EC + Ge (0.5%)	> 21	> 21	> 21
EC + Lem (0.3%)	12.01	9.32	> 21
EC + Lem (0.7%)	> 21	> 21	> 21
EC + Cit (0.5%)	> 21	> 21	> 21

[a]Shelf-life calculated from modified Gompertz model (Eq. 2). 10^7 CFU/g was used as maximal limit for shelf-life calculation of all populations
EC = Edible coating; Cin = Cinnamon; Eu = Eugenol; Pal = Palmarosa; Ge = Geraniol; Lem = Lemongrass; Cit = Citral

The three EOs applied were effective to extend the fresh-cut melon shelf-life. Nevertheless, higher concentrations of those oils were in general more effective than lower concentrations with the exception of palmarosa oil which resulted equally effective at 0.3 and 0.7%. On the other hand, the active compounds evaluated also extended the shelf-life of fresh-cut melon for more than 21 days (Table 4). According to these results, palmarosa oil appears to be the best among the EOs studied to improve the shelf-life of fresh-cut melon, due to its higher effectiveness a lowest concentration (0.3%).

3.3- Effect of edible alginate coating containing malic acid and EOs on inoculated *S.* Enteritidis population in fresh-cut melon

The use of an alginate-based edible coating containing malic acid and EOs as antimicrobial substances was effective to reduce *S.* Enteritidis population in inoculated fresh-cut melon. Immediately after coating the fresh-cut melon and before storage (t = 0 days) significant differences ($p< 0.05$) among the *S.* Enteritidis counts were observed in non-coated fresh-cut melon, coated with EC and EC containing different EOs or their active compounds (Table. 5).

Table 5.- Survival fraction of *S.* Enteritidis in inoculated fresh-cut melon with edible alginate coating including or not EOs or their active compounds and stored at 5ºC for 21 days

Treatment	Survival fraction [a] (Log_{10} CFU/g)				
	Storage time (days)				
	0	3	7	14	21
Non-coated	6.74 ± 0.17Ca	6.49 ± 0.14Ca	5.93 ± 0.25Cb	5.13 ± 0.17Cc	4.5 ± 0.1Cd
EC	5.39 ± 0.11Da	5.2 ± 0.3Dab	4.83 ± 0.19Db	4.21 ± 0.12Dc	3.66 ± 0.26Dd
EC + Cin (0.3%)	5.33 ± 0.25Ca	4.83 ± 0.15Cab	4.4 ± 0.3Cb	3.73 ± 0.19Cc	3.0 ± 0.2Cd
EC + Cin (0.7%)	4.61 ± 0.18Ba	4.6 ± 0.1Ba	4.41 ± 0.05Ba	3.6 ± 0.2Bb	2.69 ± 0.06Bc
EC + Eu (0.5%)	4.96 ± 0.06Ca	4.80 ± 0.06Cab	4.592 ± 0.021Cb	4.13 ± 0.17Cc	3.46 ± 0.07Cd
EC + Pal (0.3%)	5.06 ± 0.13Ca	4.91 ± 0.09Cab	4.74 ± 0.04Cbc	4.43 ± 0.06Cc	3.9 ± 0.2Cd
EC + Pal (0.7%)	4.91 ± 0.06CDa	4.81 ± 0.03CDab	4.59 ± 0.14CDb	4.06 ± 0.17CDc	2.78 ± 0.11CDd
EC + Ge (0.5%)	5.1 ± 0.2CDa	4.91 ± 0.05CDa	4.64 ± 0.23CDa	4.15 ± 0.22CDb	3.11 ± 0.16CDc
EC + Lem (0.3%)	5.32 ± 0.15Ba	5.03 ± 0.24Bab	4.62 ± 0.11Bb	3.74 ± 0.2Bc	2.56 ± 0.09Bd
EC + Lem (0.7%)	5.27 ± 0.27Ba	4.86 ± 0.13Bab	4.46 ± 0.07Bb	3.6 ± 0.27Bc	2.54 ± 0.24Bd
EC + Cit (0.5%)	5.26 ± 0.14Aa	4.98 ± 0.02Aab	4.77 ± 0.03Ab	4.23 ± 0.25Ac	3.84 ± 0.16Ad

[a] Mean of 4 determinations ± standard deviation.
EC = Edible coating; Cin = Cinnamon; Eu = Eugenol; Pal = Palmarosa; Ge = Geraniol; Lem = Lemongrass; Cit = Citral.

Values in the same column that are not followed by the same capital letter are significantly different ($p < 0.05$); values in the same row that are not followed by the same lower-case letter are significantly different ($p < 0.05$).

A lower initial population of *S*. Enteritidis (5.39 ± 0.11 Log_{10} CFU/g) was observed in coated fresh-cut melon with EC than in non-coated fresh-cut melon (6.74 ± 0.17 Log_{10} CFU/g). However, initial populations of *S*. Enteritidis in coated fresh-cut melon with EC containing EOs or their active compounds were lower than that found in coated fresh-cut melon with EC due probably to an increased antimicrobial effect produced by the combination of malic acid and EOs into the coating, ranging the initial population between 4.61 ± 0.18 to 5.33 ± 0.25 Log_{10} CFU/g, depending on the antimicrobial added (Table 5).

Ukuku and Fett (2004) reported a reduction of *Salmonella* population similar to our (1.35 Log CFU/g) in fresh-cut melon. These authors reduced 1.4 Log CFU/g of *Salmonella* in fresh-cut melon treated with 50 μg/ml of nisin, 2% sodium lactate and 0.02% potassium sorbate. On the other hand, a higher reduction of *Salmonella* was found by Eswaradandam et al., (2004) when evaluate the antimicrobial activity of citric, lactic, malic or tartaric acids and nisin-incorporated soy protein film against *Salmonella* Gaminara, reporting that only the incorporation of malic acid onto the soy protein film resulted in a reduction of 6 Log cycles of *Salmonella* population. But when nisin was incorporated onto the soy protein film the reduction reached was only 3.3 Log cycles. In such sense, Dawson et al., (2002) indicated that antimicrobial films are often more effective in inhibiting target microorganism when are applied to nutrient media than when are used in real food systems.

High concentrations of EOs resulted more effectives to reduce *S*. Enteritidis than low concentrations. Table 5, shows that edible coating containing 0.7% of cinnamon was the best to reduce the microorganism population in coated fresh-cut melon during the first storage hours (2.13 Log reductions) in comparison with the rest of the EOs and active compounds added, which ranged from 1.83 to 1.41 and from 1.78 to 1.48 Log reductions for EOs and their active compounds, respectively.

The incorporation of active compounds such as eugenol, geraniol and citral at 0.5% into the edible alginate coating resulted not more effective than the use of high concentrations of cinnamon, palmarosa and lemongrass oils to reduce *S*. Enteritidis population in coated fresh-cut melon. Some studies have concluded that unfractionated EOs have a greater antibacterial activity than the major components mixed, suggesting that minor components of the EOs are critical to the activity and may have a synergistic effect (Burt, 2004).

Significant reductions ($p < 0.05$) of *S*. Enteritidis population were also observed through the storage time for all treatment conditions including the control. However the reductions found in coated fresh-cut melon were higher than that observed in non-coated. Those reductions in *S*. Enteritidis observed along the storage time in non-coated fresh-cut melon could be due to effects such as storage temperature (5ºC) and competitive microflora (native flora), whereas the reductions observed through the time in coated fresh-cut melon are due to combined effects of antimicrobials added and those other factors indicated (Table 5). Those results differ from that reported by Golden et al., (1993) who found little or any decrease in *Salmonella* population in fresh-cut Cantaloupe, Watermelon and Honeydew melons stored at 5ºC for 24h. This latter phenomenon could be explained by the storage time used, since those authors used 24h of storage and we used 21 days of storage.

Concentrations of 0.3% (4.20 Log cycles) and 0.7% (4.18 Log cycles) of lemongrass and 0.7% of cinnamon (4.05 Log cycles) caused the highest reductions of the *S*. Enteritidis population at 21 of storage (Table 5). In addition, it is possible to observe in Table 5 that coatings with geraniol or eugenol at 0.5% had similar antimicrobial activity over *S*. Enteritidis population; whereas, the addition of 0.5% of citral to the coating solution caused a minor reduction of *S*. Enteritidis population.

According to the literature, combinations of treatments are more effective to reduce the *Salmonella* populations in whole and fresh-cut melon than single treatments (Ukuku and Fett 2004; Ukuku et al., 2005). The rind roughness of some melon varieties as cantaloupe and honeydew can be the cause of a better attachment of *Salmonella* in whole melons, whereas the high pH of this fruit may be the responsible of the survival of the microorganisms in fresh-cut melon. A pH of 6.06 ± 0.01 was found in the melon flesh used in this study, which help to the survival of *S*. Enteritidis in the processed fruit. On the other hand, since previous researches have demonstrated the effectiveness of the EOs and malic acid to inactivate up to 5 cycles of *S*. Enteritidis in melon juice (Raybaudi-Massilia et al., 2006) and fresh-cut "Flor de invierno" pears (Unpublished results) respectively, we consider that an interaction between the constituents of the edible coating, malic acid and EOs caused a decrease of the effectiveness of these compounds as was also reported by Eswaradandam et al., (2004) who indicated that a possible interaction between nisin and malic acid occurred when evaluate the antimicrobial

activity of citric, lactic, malic or tartaric acids and nisin-incorporated soy protein film against *Salmonella* Gaminara.

3.4- Effect of edible alginate coating containing malic acid and EOs over the physicochemical parameters of fresh-cut melon

3.4.1-Changes in headspace gas composition

Oxygen consumption and carbon dioxide production in packed fresh-cut melon non-coated and coated with EC or EC with EOs or their active compounds is showed in Fig. 4 and 5. Significant differences ($p<0.05$) were found in the pattern oxygen consumption and carbon dioxide production between non-coated and coated fresh-cut melon. Higher oxygen consumption and carbon dioxide production were observed for non-coated fresh-cut melon, observing the bigger changes from 7 days of storage (Fig. 4 and 5). The consumption of oxygen and carbon dioxide production in fresh-cut melon can be explained in general by the respiration of the tissue, which continue being a living tissue even after cutting, as well as by the respiration of microbial flora. A diminution of oxygen levels into the fresh-cut melon package lead to an anaerobic transformation of the melon sugars into alcohol and CO_2 by the native flora, increasing the concentration of those gases into the trays as was observed in non-coated fresh-cut melon. Similar results were reported by Oms-Oliu et al., (2006) who observed a drastic diminution of oxygen concentration into the trays of fresh-cut "Piel de Sapo" melon and an increase of carbon dioxide production also indicating that production of that last gas was significantly affected by the melon ripeness. Those authors indicated that enhanced CO_2 production may be due to microbial growth and a general deterioration of the tissue fresh-processed melon. Similar pattern of oxygen consumption and carbon dioxide production have been reported in fresh-cut apples and pears stored at refrigeration temperature (Soliva-Fortuny et al., 2002a, 2002b and 2004; Rojas-Graü et al., 2007c; Raybaudi-Massilia et al., 2007).

Light decreases of oxygen concentration and carbon dioxide were found in coated fresh-cut melon, influencing significantly the type and concentration of EOs used and their active compounds (Fig. 4 and 5). In this way, EC was effective to avoid a drastic reduction of oxygen concentration and carbon dioxide production as occurred in non-coated fresh-cut melon. That result is a direct consequence of the inhibition of the native microflora growth achieved with the use of that specific coating as is shown in Fig. 1, 2 and 3 as well as by the coating influence over the oxygen diffusion between the fruit and environment as was also indicated by

Baldwin et al., (1996). In addition, Wong et al., (1994) indicated that the diffusion of the headspace oxygen to the tissue apple pieces was inhibited by the high oxygen resistance of the coating. However, different results

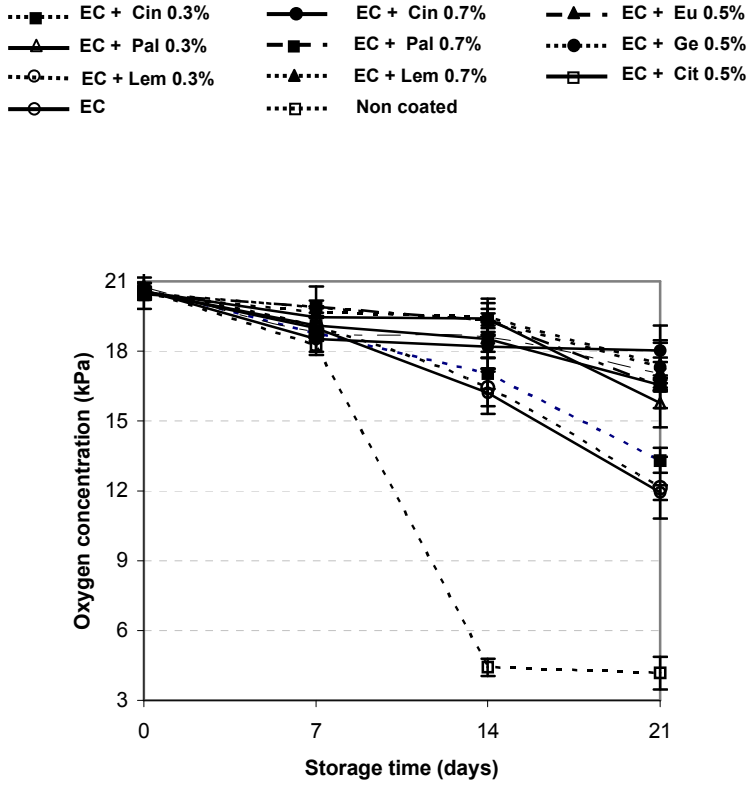


Figure 4.- Changes in oxygen concentration (kPa) in fresh-cut melon pieces with edible alginate coating (EC) including or not EOs of cinnamon (Cin), Palmarosa (Pal) and Lemongrass (Lem) at 0.3 and 0.7% or their main active compounds: eugenol (Eu), geraniol (Ge) and citral (Cit) at 0.5%. The values represented are mean of 4 determinations ± standard deviation.

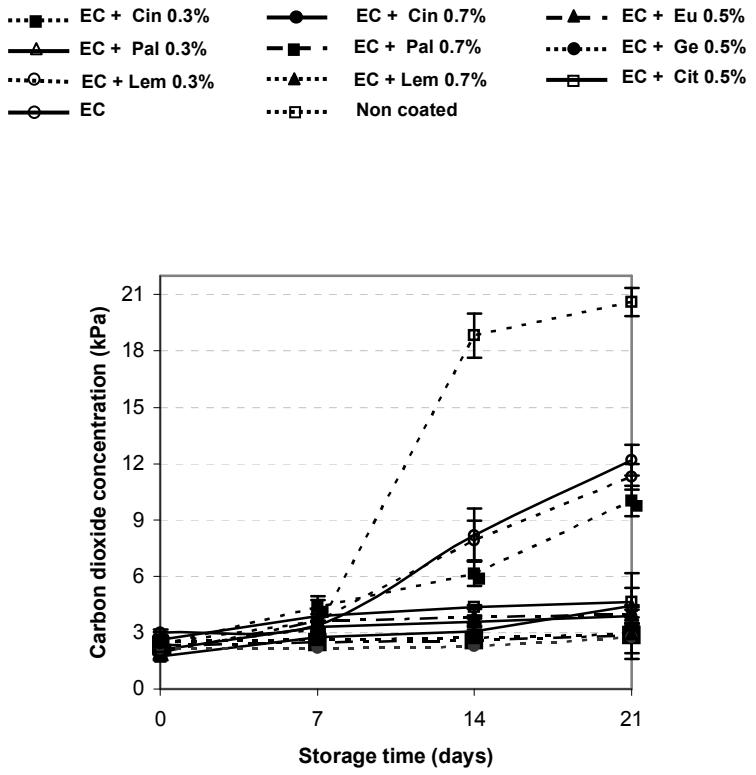


Figure 5.- Changes in carbon dioxide concentration (kPa) in fresh-cut melon pieces with edible alginate coating (EC) including or not EOs of cinnamon (Cin), Palmarosa (Pal) and Lemongrass (Lem) at 0.3 and 0.7% or their active compounds: eugenol (Eu), geraniol (Ge) and citral (Cit) at 0.5%. The values represented are mean of 4 determinations ± standard deviation.

were showed by Rojas-Graü et al., (2007d) who did not find significant differences ($p<0.05$) between the O_2 consumption and CO_2 production in fresh-cut apples non-coated or coated with edible alginate or gellan based coatings. Those differences may be consequence of the formulation coatings since our formulation include a crosslink with calcium lactate solution containing malic acid which have an antimicrobial effect over native flora, causing a diminution in O_2 consumption and CO_2 production as previously demonstrated (Raybaudi-Massilia et al., 2007). On the other hand, the incorporation of EOs and their active compounds into the edible coating caused minor oxygen consumption and carbon dioxide production as consequence of a greater inhibition of the microflora growth caused probably by combined action of malic acid and EOs or their active compounds as was demonstrated in this study (Fig. 1, 2 and 3) and possibly to a major resistance by the coating to gases diffusion caused by the lipophilic nature of the added substances. Thus, lower concentration of cinnamon (0.3%) and lemongrass (0.3%) showed a diminution of oxygen concentration and carbon dioxide production greater than the higher concentration (0.7%) of those EOs, whereas palmarosa at 0.3 and 0.7% not showed significant differences (Fig. 4 and 5). On the other hand, significant differences ($p<0.05$) among the behaviour of those gases were not found when the three active compounds (eugenol, geraniol and citral) were compared. Similar results were reported by Rojas-Graü et al., (2007a) who indicated that lower oxygen consumption and carbon dioxide production were observed in coated fresh-cut apple when EOs were added to edible alginate-apple pure coatings. In addition, those authors indicated that the behaviour of those gases was significantly affected by the EOs concentrations, being the highest EOs concentrations more influential than those lowest concentrations.

Ethanol is an indicator of the degree of anaerobic fermentation caused by microbial growth and its accumulation can takes place when internal atmosphere is affected and gas exchange is restricted as occur when edible coatings are used (Park et al., 1994). An increase in the production of this gas along the storage time was observed for all treatment conditions (Fig. 6). Nevertheless, the production of that gas was much higher in non-coated than coated melon pieces as a consequence of a higher microbial growth during storage. In such sense, melon sugars fermentation caused by yeast could explain that fact, since higher populations of yeast and moulds were observed in those non-coated melon pieces than in those coated melon pieces (Fig. 3). On the other hand, differences significant in

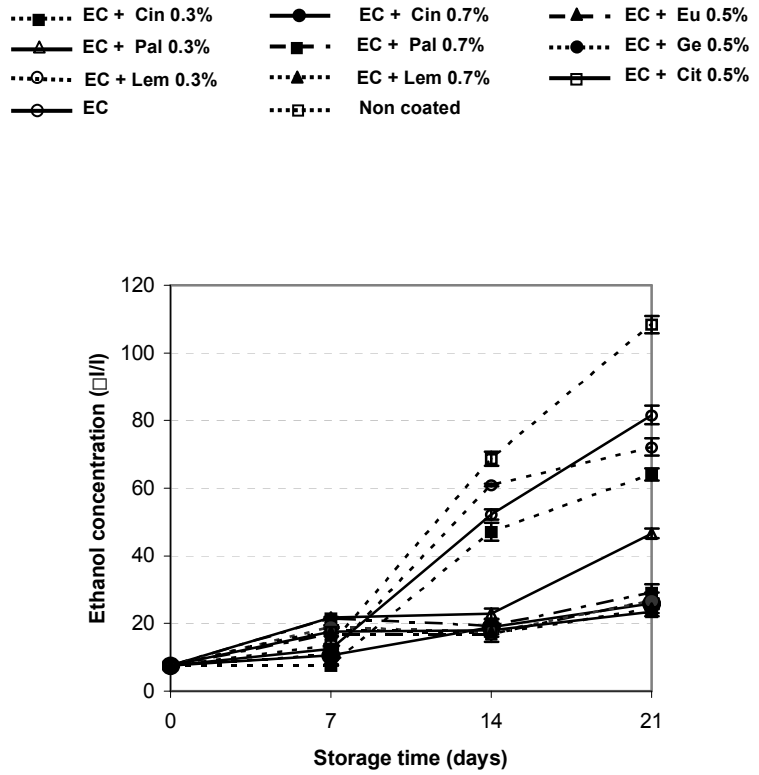


Figure 6.- Changes in Ethanol concentration (μl/l) in fresh-cut melon pieces with edible alginate coating (EC) including or not EOs of cinnamon (Cin), Palmarosa (Pal) and Lemongrass (Lem) at 0.3 and 0.7% or their active compounds: eugenol (Eu), geraniol (Ge) and citral (Cit) at 0.5%. The values represented are mean of 4 determinations ± standard deviation.

ethanol production were only observed when cinnamon (0.7%), palmarosa (0.3 and 0.7%), lemongrass (0.7%), eugenol (0.5%), geraniol (0.5%) and citral (0.5%) were added to edible coatings, showing a lower production of this gas in comparison with the rest of the treatments, demonstrating that EOs in high concentrations and their active compounds can limit the ethanol production in coated melon pieces. Those results are a consequence of the combined antimicrobial effects that malic acid and EOs or their active compounds have over the native microflora. A similar pattern of ethanol production along the storage time was observed by Rojas-Graü, et al., (2007a) in coated fresh-cut apple, indicating that fresh-cut apple coated with alginate-apple pure without EOs reached higher levels of ethanol at 21 of storage than those fresh-cut apples coated with the same base coating and lemongrass (1.5%) and oregano (0.3%) oils included.

Due to decrease of oxygen and increase of carbon dioxide and ethanol concentrations shelf-life of non-coated fresh-cut melon was limited to 7 days, whereas an extension until 14 -21 days was achieved when EC with or without EOs was applied to fresh-cut melon.

3.4.2.-Firmness changes

Significant differences ($p < 0.05$) were found between non-coated and coated fresh-cut melon immediately after applying the coating and before to storage ($t = 0$ days) as a consequence of the calcium added to the crosslink solution. In such sense, others researchers have reported that calcium chloride can maintain the firmness in fruits when is incorporated into the edible coatings (Olivas and Barbosa-Cánovas, 2005; Rojas-Graü et al., 2007d). On the other hand, the incorporation of EOs and their active compounds into the coating have a significant ($p> 0.05$) effect over the fresh-cut melon firmness, decreasing significantly when higher concentrations of EOs were used (Table 6). In this way, palmarosa, lemongrass and their active compounds geraniol and citral affected the firmness of the fresh-cut melon in higher proportion than the others substances added (Table 6). That result may be a consequence of the action of the EOs over the cell tissue of the fruit, which possibly undergo structural changes that affect directly the fruit firmness. Palmarosa, lemongrass and cinnamon leaf have different composition containing distinct active compounds such as geraniol, citral and eugenol respectively (Wright, 1999; Velluti et al., 2003; Prashar et al., 2003). However, EOs have other secondary compounds which have a synergistic effect with those actives compounds as have been indicated by Burt, (2004). In this way, it is

Table 6.- Firmness of fresh-cut melon pieces with edible alginate coating including or not EOs or their active compounds and stored at 5ºC for 21 days.

Treatment	Firmness[α] Storage time (days)				Shelf-life[δ] (days)
	0	7	14	21	
Non-coated	4.03 ± 0.24ABCa	3.77 ± 0.34ABCa	3.17 ± 0.51ABCa	3.13 ± 0.01ABCa	< 14
EC	4.6 ± 0.3Ga	4.52 ± 0.17Ga	4.34 ± 0.11Ga	4.30 ± 0.02Ga	> 21
EC + Cin (0.3%)	4.68 ± 0.08Ga	4.54 ± 0.35Ga	4.52 ± 0.01Ga	4.26 ± 0.41Ga	> 21
EC + Cin (0.7%)	4.1 ± 0.5BCa	3.83 ± 0.19BCa	3.6 ± 0.3BCa	3.0 ± 0.5BCa	> 14
EC + Eu (0.5%)	4.4 ± 0.8FGa	4.15 ± 0.11FGa	4.2 ± 0.8FGa	3.9 ± 0.7FGa	> 21
EC + Pal (0.3%)	4.1 ± 0.5ABDa	3.6 ± 0.2ABDab	3.52 ± 0.21ABDb	3.39 ± 0.33ABDb	> 21
EC + Pal (0.7%)	3.64 ± 0.07Ea	2.68 ± 0.17Eb	2.53 ± 0.47Eb	2.49 ± 0.06Eb	< 7
EC + Ge (0.5%)	3.82 ± 0.25DEa	3.32 ± 0.28DEa	2.66 ± 0.10DEb	2.54 ± 0.14DEb	> 7
EC + Lem (0.3%)	4.51 ± 0.09CFa	3.96 ± 0.18CFab	3.95 ± 0.18CFab	3.35 ± 0.40CFb	> 21
EC + Lem (0.7%)	3.91 ± 1.13ADa	3.18 ± 0.46 ADa[β]	2.84 ± 0.08ADa	2.7 ± 0.9ADa	< 7
EC + Cit (0.5%)	3.61 ± 0.20DEa	3.09 ± 0.61DEa	2.70 ± 0.32DEa	2.69 ± 0.35DEa	< 7

[α] Mean of 10 determinations ± standard deviation

[β] Reference point considered sensorially undesirable

[δ] Shelf-life based on firmness reference point

EC = Edible coating; Cin = Cinnamon; Eu = Eugenol; Pal = Palmarosa; Ge = Geraniol; Lem = Lemongrass; Cit = Citral

Values in the same column that are not followed by the same capital letter are significantly different ($p < 0.05$); values in the same row that are not followed by the same lower-case letter are significantly different ($p < 0.05$).

important to indicate that palmarosa and lemongrass have some secondary compounds in common such as geraniol (in the lemongrass case) and geranyl acetate which is found as secondary compound in both oils palmarosa and lemongrass, which can explain why this two EOs have similar behaviour over the fresh-cut melon firmness.

Enzymes and substrates are normally located in different cellular compartments and their motion is actively regulated. However, destruction of surface cells and injury stress of underlying tissues occur during processing of the fruit promoting the exit of some enzymes such as pectic enzymes which cause firmness changes (Knee, 1973). Enzymatic reactions can also cause sensory deterioration such as off-flavour and discoloration (Varoquaux and Wiley, 1994). In this work, a diminution of the fresh-cut melon firmness occurred through the storage time in non-coated fresh-cut melon, reaching a value of 3.13 ± 0.01 N at 21 days of storage (Table 6) in comparison with

coated EC fresh-cut melon which reached a value of 4.30 ± 0.02 N. On the other hand, decreases in firmness along the time were also observed in fresh-cut melon coated with EC including EOs or their active compounds depending on the kind of compound and concentration (Table 6). Firmness was considered as sensorially undesirable from a value of 3.18 N corresponding to fresh-cut melon coated with EC + 0.7% lemongrass (at 7 days) due to its soft aspect in comparison with a fresh-cut melon. In this sense, shelf-life of coated and non-coated fresh-cut melon was considered in the base of this reference point. Edible coating resulted effective to extend the shelf-life from a textural point of view by more than 21 days (Table 6) demonstrating that EC was a good calcium lactate carrier to the fruit. On the other hand, shelf-life of fresh-cut melon coated with EC and EOs or their active compounds added varied, being the fresh-cut melon coated with EC + palmarosa (0.7%), lemongrass (0.7%), or citral (0.5%) the most affected since showed a shelf-life lower than 7 days (Table 6).

3.4.3.-Color changes

Significant differences ($p < 0.05$) were not found after comparing whiteness index (WI) in non-coated fresh-cut melon and coated with EC immediately after processed (T = 0 days) (Table 7). However, significant changes in this parameter were observed when EOs and their active compounds were added (Table 7). Higher concentrations of EOs affected more whiteness of fresh-cut melon than the lower concentrations (Table 7). Likewise, eugenol and geraniol at 0.5% caused a higher diminution of WI than citral at 0.5%. A depletion of whiteness through the storage time was also observed in both coated and non-coated fresh-cut melon (Table 7). However, that reduction in WI was significant only when lower concentrations of EOs were used indicating that this decrease was gradual along the storage time. Nevertheles, in fresh-cut melon with edible coating including EOs in higher concentrations that reduction occurred during the first hours of storage (t = 0 days) since significant differences ($p< 0.05$) were not found through the storage time.

Other parameter which was determined as indicative of color changes of fresh-cut melon in this study was C* which was calculated through the equation 4. Significant differences ($p< 0.05$) for C* values among non-coated fresh-cut melon and coated with EC were not detected at t = 0 days, however significant differences were observed for this parameter when EOs or their active compounds were added to the coating solution. On the other hand, a significant diminution ($p<0.05$) of this parameter along the storage time was found. Thus, fresh-cut melon with edible coating including

Table 7.- Changes in Whiteness index of fresh-cut melon pieces with edible alginate coating including or not EOs or their active compounds and stored at 5ºC for 21 days.

Treatment	Whiteness index $^{\alpha}$(WI)				Shelf-life$^{\delta}$ (days)
	0	7	14	21	
Non-coated	66.7 ± 0.7 Aa	66.6 ± 0.9 Aa	65.3 ± 0.4 Ab	64.8 ± 0.3 Ab	> 21
EC	66.7 ± 0.2 Aa	65.4 ± 0.3 Aa	62.9 ± 0.7 Ab	62.4 ± 0.9 Ab	> 21
EC + Cin (0.3%)	64.6 ± 0.8 Ba	63.3 ± 0.7 Bab	62.8 ± 0.4 Bb	60.2 ± 0.4 Bc	> 14
EC + Cin (0.7%)	63.02 ± 0.01Ca	62.2 ± 0.9 Ca	61.76 ± 0.11$^{\beta}$ Ca	61.7 ± 0.4 Ca	> 14
EC + Eu (0.5%)	62.7 ± 0.5 Ca	62.6 ± 0.6 Ca	62.2 ± 0.8 Ca	61.7 ± 0.6 Ca	> 14
EC + Pal (0.3%)	64.6 ± 0.4 Ba	64.41 ± 0.09 Bab	62.7 ± 0.4 Bb	62.71 ± 0.01 Bb	> 21
EC + Pal (0.7%)	63.02 ± 0.21 Ca	62.6 ± 0.4 Ca	62.8 ± 0.5 Ca	61.58 ± 0.09 Ca	> 14
EC + Ge (0.5%)	63.41 ± 0.20 Ca	63.82 ± 0.22 Ca	63.7 ± 0.9 Ca	62.6 ± 0.4 Ca	> 21
EC + Lem (0.3%)	64.60 ± 0.20 Ba	65.01 ± 0.20 Ba	63.39 ± 0.18 Bb	62.3 ± 0.9 Bc	> 21
EC + Lem (0.7%)	62.9 ± 0.6 Ca	62.41 ± 0.21 Ca	62.1 ± 0.5 Ca	61.58 ± 0.02 Ca	> 14
EC + Cit (0.5%)	65.6 ± 0.4 Da	63.7 ± 0.6 Db	62.5 ± 0.3 Db	60.6 ± 0.8 Dc	> 14

$^{\alpha}$ **WI** data calculated from Eq. 3 are the mean of 30 determinations ± standard deviation

$^{\beta}$ Reference point considered sensorially undesirable

$^{\delta}$ Shelf-life based on whiteness reference point

EC = Edible coating; Cin = Cinnamon; Eu = Eugenol; Pal = Palmarosa; Ge = Geraniol; Lem = Lemongrass; Cit = Citral

Values in the same column that are not followed by the same capital letter are significantly different ($p<0.05$); values in the same row that are not followed by the same lower-case letter are significantly different ($p < 0.05$).

essential oil of palmarosa at 0.3 and 0.7% were the more affected at 21 days of storage. Changes in WI and C* was also reported by Oms-Oliu et al., (2006) and Aguayo et al., (2004) in "Piel de sapo" melon and others varieties, indicating those authors that WI decreased when translucency injury increased on fresh-processed melon, as a consequence of a physiological disorder characterized by dark and glassy flesh.

Fresh-cut melon pieces with whiteness and chroma values ≤ to 61.76 and 6.3 respectively were considered as undesirables since a translucent look was observed in those cases in comparison with fresh-cut melon. In this sent, shelf-life of coated and non-coated fresh-cut melon was considered based on that reference point (Table 7 and 8). The WI and C* values of non-coated fresh-cut melon and coated with EC showed a slight

Table 8.-Changes in Chroma of fresh-cut melon pieces with edible alginate coating including or not EOs or their active compounds and stored at 5ºC for 21 days

Treatment	Chroma [α] (C*)				Shelf -life [δ]
	0	7	14	21	
Non-coated	8.72 ± 0.02 AEa	8.65 ± 0.09 AEa	8.0 ± 0.3 AEa	7.89 ± 0.26 AEa	> 21
EC	8.70 ± 0.01 AEa	8.62 ± 0.15 AEa	8.41 ± 0.13 AEa	8.20 ± 0.05 AEa	> 21
EC + Cin (0.3%)	8.0 ± 0.3 Ba	7.78 ± 0.13 Ba	7.03 ± 0.14 Bb	6.9 ± 0.23 Bb	> 21
EC + Cin (0.7%)	7.8 ± 0.1 Ba	7.7 ± 0.3 Bab	7.46 ± 0.05 Bbc	7.3 ± 0.5 Bc	> 21
EC + Eu (0.5%)	8.14 ± 0.18 BCa	8.09 ± 0.35 BCa	7.4 ± 0.4 BCb	6.6 ± 0.6 BCc	> 21
EC + Pal (0.3%)	8.507 ± 0.07 CDa	7.413 ± 0.43 CDb	6.58 ± 0.22 CDc	5.37 ± 0.45 CDd	> 14
EC + Pal (0.7%)	7.76 ± 0.09 Ba	7.4 ± 0.3 Ba	6.3 ± 0.7 [β] Bb	4.99 ± 0.06 Bc	> 14
EC + Ge (0.5%)	8.77 ± 0.09 AEa	6.61 ± 0.04 AEb	6.49 ± 0.13 AEb	6.3 ± 0.5 AEb	> 21
EC + Lem (0.3%)	8.64 ± 0.31 AEa	8.1 ± 0.6 AEab	7.49 ± 0.13 AEb	6.2 ± 0.4 AEc	> 14
EC + Lem (0.7%)	7.79 ± 0.10 Ba	7.504 ± 0.4 Bab	6.98 ± 0.28 Bb	6.1 ± 1.0 Bc	> 14
EC + Cit (0.5%)	8.90 ± 0.29 Ea	7.82 ± 0.07 Eab	7.79 ± 0.26 Eb	6.48 ± 0.12 Ec	> 21

[α] C* data calculated from Eq. 4 are the mean of 30 determinations ± standard deviation

[β] Reference point considered sensorially undesirable

δ Shelf-life based on chroma reference point

EC = Edible coating; Cin = Cinnamon; Eu = Eugenol; Pal = Palmarosa; Ge = Geraniol; Lem = Lemongrass; Cit = Citral

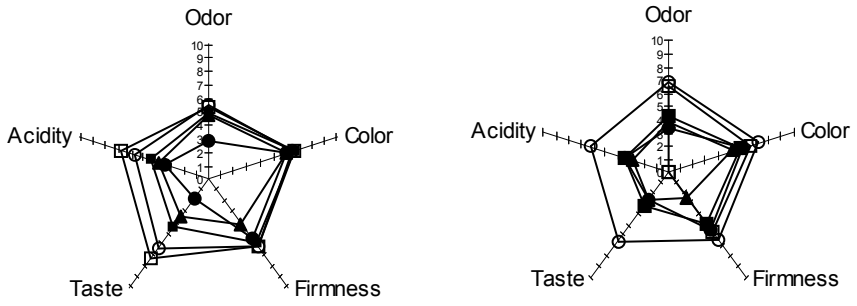
Values in the same column that are not followed by the same capital letter are significantly different ($p < 0.05$); values in the same row that are not followed by the same lower-case letter are significantly different ($p < 0.05$).

ecrease though the storage time, therefore shelf-life of those cases was considered > 21 days. However, the incorporation of EOs or their active compounds to edible coating affected shelf-life of fresh-cut melon being in some cases lower than 21 days (Table 7 and 8)

3.4.4.-Sensory evaluation of fresh-cut melon

Storage time did not show an important effect on the evaluated sensory characteristics (odour, color, firmness, taste and acidity) since statistically significant differences ($p < 0.05$) were not found between the values obtained at 0 and 8 days, indicating that changes throughout the storage time did not appear to be perceived by the panellists. Fresh-cut melon coated with EC showed similar acceptation of non-coated fresh-cut melon since significant differences ($p<0.05$) were not found when statistical comparison through an multifactor ANOVA was made, indicating that edible based coating with malic acid added

may be a good preservation alternative since maintain the sensory characteristic similar to non-coated fresh-cut melon in addition to inhibit the microbial growth
and gas production that affect the fruit quality. That result is according to Debeaufort, Quezada-Gallo & Voilley (1998) who indicated that edible coatings use can contribute to reduce the loss of volatiles compounds from the food avoiding thus the partial or total loss of food flavours maintaining in this way their quality. Contrary to that result, significant differences ($p<0.05$) in fresh-cut melon odour, taste and firmness depending on the essential oil added to edible coating were detected (Fig. 7). Odour and taste characteristic were significantly affected ($p<0.05$) by incorporation of cinnamon oil since lower acceptation of fresh-cut melon was observed when this essential oil was added in comparison with palmarosa or lemongrass.



t = 0 days **t = 8 days**

Figure 7.- Influence of storage time on sensory characteristic of fresh-cut melon pieces with edible alginate coating including EOs of cinnamon (●), Palmarosa (■) and Lemongrass (▲) at 0.7%. Controls with edible coating without essential oils (○) and fresh-cut melon non-coated (□) also were evaluated. The values are mean of 30 determinations. Taste and acidity determination at 8 days in fresh-cut melon non-coated were not carried out due to its high microbiological load.

Likewise, firmness was significantly affected by the incorporation of lemongrass into the edible coating from the sample preparation day (t = 0 days) intensifying that negative effect during the storage time.

4- CONCLUSIONS

Alginate-based edible coating containing malic acid improved the shelf-life of fresh-cut melon from microbiological and physicochemical points of view in comparison with non-coated fresh-cut melon. In addition, a reduction of inoculated *S.* Enteritidis population in fresh-cut melon using EC was achieved, improving thus, the fruit safety. On the other hand, the incorporation of EOs or their active compounds into EC prolonged by more time the microbiological shelf-life of fresh-cut melon probably as a consequence of an enhanced antimicrobial effect mainly caused by the combination of malic acid and EOs into the coating. However, some physicochemical characteristics of the melon pieces were affected with those EOs. According to the results obtained in this study, palmarosa oil at 0.3% incorporated into EC appear to be a promising conservation alternative for fresh-cut melon, since has a good acceptation by panellists, maintains the quality parameters of the fruit, inhibits the native flora growth and reduces *S.* Enteritidis population.

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DISCUSION GENERAL

Para la realización de esta investigación acerca del uso de sustancias antimicrobianas naturales en combinación con compuestos estabilizadores de la calidad para controlar microorganismos patógenos y extender la vida útil de frutas frescas cortadas se seleccionaron tres tipos de fruta: manzanas "Fuji" (*Malus domestica* Borkh), peras "Flor de invierno" (*Pyrus communis* L.) y melones "Piel de Sapo" (*Cucumis melo* L.) por sus excelentes propiedades fisicoquímicas y sensoriales.

Por otra parte, sustancias naturales antimicrobianas tales como ácido málico, diferentes aceites esenciales y sus principales compuestos activos fueron seleccionados para el presente estudio de acuerdo a la bibliografía y pruebas preliminares en el laboratorio, basadas principalmente en su efectividad contra microorganismos patógenos como *L. monocytogenes*, *Salmonella* serovars y *E. coli* O157:H7 así como también en su compatibilidad con frutas y sus derivados.

Determinación de las concentraciones mínimas inhibitorias (CMIs) y mínimas bactericidas (CMBs) de ácido málico contra *Listeria monocytogenes*, *Salmonella* Enteritidis y *Escherichia coli* O157:H7 en zumos de frutas.

Ácido málico fue seleccionado entre los ácidos orgánicos para ser utilizado en este estudio por estar presente en forma natural en las tres frutas seleccionadas manzana, pera y melón (Eisele y Drake, 2005; Binnig y Possmann, 1993; Luh y El-Tinay, 1993; Lamikanra y col., 2000), por su potencial para impartir excelentes propiedades sensoriales a los productos de fruta (Raju y Bawa, 2006), así como también por haber mostrado mayor actividad antimicrobiana que otros ácidos evaluados contra *L. monocytogenes*, *S.* Enteritidis y *E. coli* O157:H7 tanto en estudios realizados en nuestro laboratorio como en estudios efectuados por otros investigadores (Eswaranandam y col., 2004). Una vez seleccionado el ácido se procedió a determinar las concentraciones mínimas inhibitorias (CMIs) y mínimas bactericidas (CMBs) contra *L. monocytogenes*, *S.* Enteritidis y *E. coli* O157:H7 en zumo de manzana, pera y melón almacenados a 5, 20 y 35ºC.

Los resultados demostraron que las CMIs y CMBs de ácido málico contra *L. monocytogenes*, *S.* Enteritidis y *E. coli* O157:H7 fueron significativamente ($p < 0.05$) afectadas por la temperatura y tiempo de almacenamiento, tipo de zumo y microorganismo. Así, los zumos de manzana, pera y melón inhibieron el crecimiento de los tres patógenos a 5ºC durante 120 h de almacenamiento, mientras que a 20 y 35ºC solo los zumos de manzana y pera mostraron este efecto sobre los microorganismos, siendo necesarias concentraciones de 0.2, 0.2 y 0,4% de ácido málico para inhibir el crecimiento de *L. monocytogenes*, *S.* Enteritidis y *E. coli* O157:H7, respectivamente en zumo de melón almacenado a 20 y 35ºC. Estos resultados están en concordancia con los reportados por otros investigadores, quienes encontraron que *L. monocytogenes* y *E. coli* O157:H7 sobrevivieron pero no crecieron en zumo o sidra de manzana almacenados a diferentes temperaturas (Zhao y col., 1993; Miller y Kaspar, 1994; Yuste y Fung, 2002; Ceylan y col., 2004).

Mayores concentraciones de ácido málico fueron generalmente requeridas para reducir o inactivar (CMBs) las poblaciones de *L. monocytogenes*, *S.* Enteritidis y *E. coli* O157:H7 en zumo de melón (pH 5,45) y pera (pH 4,60) que en zumo de manzana (pH 3,94), indicando que el pH inicial del medio de crecimiento es un factor fundamental que debe ser considerado cuando se usan los ácidos orgánicos como agentes antimicrobianos en alimentos. Así, concentraciones de 0.2, 0.4 y 0.6% de ácido málico en zumos de manzana, pera y melón, respectivamente, almacenados a 20 y 35ºC fueron requeridas para reducir las poblaciones de *L. monocytogenes* y *S.* Enteritidis en más de 1ciclo logarítmico, mientras que para reducir la población de *E. coli* O157:H7 en estos zumos almacenados a 20ºC (0.6, 1 y 2%) y 35ºC (0.4, 0.6 y 0.8 %) concentraciones más altas fueron necesarias. Sin embargo, a 5ºC las CMBs requeridas para reducir las poblaciones de cada uno de estos microorganismos en zumos de manzana y pera fueron similares mientras que en zumo de melón fueron mayores.

Por otra parte, la temperatura y el tiempo de almacenamiento ejercieron efectos significativos ($p < 0.05$) sobre las concentraciones mínimas para reducir o inactivar en más de 5 ciclos logarítmicos las poblaciones de *L. monocytogenes*, *S.* Enteritidis y *E. coli* O157:H7, siendo el ácido málico más efectivo a 20 y 35ºC que a 5ºC sobre estos patógenos en zumos de frutas. En general, concentraciones de 2, 2,5 y 2,5 % de ácido málico fueron necesarias para reducir las poblaciones de los tres patógenos en más de 5 ciclos logarítmicos en zumos de manzana, pera y melón, respectivamente, almacenados a 5ºC por 24h, mientras que menores

concentraciones de este ácido fueron suficientes para alcanzar este efecto sobre los patógenos a 20ºC (0.8, 1.5 y 2.5%) y 35ºC (0.8, 1.5 y 1.5%) en el mismo tiempo (24h). Una mayor fluidez de la membrana celular de los microorganismos a altas temperaturas (> 20ºC) puede haber favorecido la entrada del ácido málico al interior celular, ya que a bajas temperaturas, los fosfolípidos se encuentran empacados en un gel de estructura rígida, mientras que a altas temperaturas ellos están menos ordenados y la membrana tiene una estructura líquida-cristalina (Aronsson y Rönner, 2001).

Así mismo el efecto bactericida del ácido málico sobre *L. monocytogenes*, *S.* Enteritidis y *E. coli* O157:H7 varió dependiendo del tiempo de almacenamiento, aumentando la acción bactericida del ácido málico en zumos de manzana, pera y melón a medida que aumentó el tiempo de almacenamiento a 5ºC.

La resistencia al ácido málico de los tres patógenos estudiados varió significativamente dependiendo de las características de la estructura de sus membranas (condición Gram) y de su ácido tolerancia, resultando *L. monocytogenes* (bacteria Gram positiva) más sensible que *S.* Enteritidis (bacteria Gram negativa), quien a su vez fue más sensible al ácido málico que *E. coli* O157:H7 (bacteria Gram negativa). Otros investigadores han reportado también una mayor ácido tolerancia de *E. coli* O157:H7 en comparación con otros microorganismos (Miller y Kaspar, 1994; Benjamin y Datta, 1995: Arnold y Kaspar, 1995: Meng y col., 2001; Ceylan y col., 2004; Ingham y col., 2006). En tal sentido, Lin y col. (1996) indicó que tres sistemas están envueltos en la ácido tolerancia de *E. coli* O157:H7, incluyendo un sistema oxidativo ácido-inducido, un sistema arginina-dependiente ácido inducido y un sistema glutamato-dependiente.

En general, la actividad antimicrobiana de los ácidos orgánicos ha sido atribuida a una reducción del pH del medio de crecimiento, disminución del pH interno de la célula microbiana como consecuencia de la ionización de las moléculas no disociadas de ácido que han entrado a la célula, interrupción del transporte de sustratos por alteración de la permeabilidad de la membrana celular o reducción de la fuerza protón motriz y quelación de iones metálicos esenciales para el crecimiento microbiano (Doores 1993; Stratford y Eklund 2003; Eswaranandam y col., 2004).

El ácido málico es un ácido orgánico de baja solubilidad lipídica (-1.26 Log octanol/agua) (Leo y col., 1971) y en consecuencia su entrada a la célula puede ser limitada, debido a que la membrana celular es impermeable a compuestos polares (Lücke, 2003). Así pues, una

disminución del pH del medio de crecimiento podría explicar su acción antimicrobiana como sugirieron Beuchat y Golden (1989). Sin embargo, otros autores han encontrado que la efectividad de los ácidos orgánicos puede variar dependiendo de su peso molecular (Eswaranandam y col., 2004), indicando que las pequeñas moléculas no disociadas de ácido málico (134,09 Dalton) y ácido láctico (90,08 Dalton) pueden entrar en las células bacterianas fácilmente disminuyendo el pH interno de la célula, mostrando una mayor actividad antimicrobiana que las moléculas largas no disociadas de los ácidos cítrico (192,13 Dalton) y tartárico (150,09 Dalton).

De acuerdo a nuestros resultados, una simple reducción del pH del medio podría explicar el mecanismo de acción del ácido málico, ya que mayores concentraciones de éste ácido fueron necesarias para reducir o inactivar los tres patógenos en zumo de pera y melón que en zumo de manzana. Sin embargo, una disminución del pH intracelular debida a la entrada del ácido málico a la célula bacteriana podría explicar mejor la acción antimicrobiana del mismo, ya que una mayor efectividad del ácido málico contra estas bacterias fue observada en zumo de manzana que en zumo de pera y melón como consecuencia de que el pH de zumo de manzana (3,94) está más cercano al valor de pKa de éste ácido (3,40) que el pH de zumo de pera (4,60) o melón (5,45), implicando una menor disociación del ácido y por consiguiente una mayor actividad antimicrobiana. En tal sentido, se puede señalar que es bien conocido que la actividad antimicrobiana de los ácidos orgánicos es dependiente directamente de su forma no disociada ya que ésta es la condición que le permite su entrada a las células microbianas.

Los estudios de microscopía electrónica de transmisión (TEM) realizados a células de *L. monocytogenes*, *S.* Enteritidis y *E. coli* O157:H7 cultivadas en caldo triptona de soja y zumo de manzana, pera y melón, tratados o no con ácido málico permitieron dilucidar el mecanismo de acción antimicrobiana del ácido málico. Así, a pesar de las diferencias existentes entre la estructura de la membrana celular de bacterias Gram positivas (*L. monocytogenes*) y Gram negativas (*S.* Enteritidis y *E. coli* O157:H7), observamos que el ácido málico entró a las células bacterianas causando importantes daños en el citoplasma de la célula, donde ocurrió una aglutinación del material celular sin aparentes daños en la membrana. Estos resultados demostraron que el pH intracelular disminuyó como consecuencia de la entrada de pequeñas moléculas no disociadas de ácido málico a la célula, que se vieron forzadas a disociarse debido al pH cercano a la neutralidad existente en el interior celular y causaron la liberación de protones que ocasionó la aglutinación del material citoplasmático

observada. Dadas las diferencias entre la estructura de la membrana celular de bacterias Gram positivas y Gram negativas podríamos indicar que el ácido málico pasó a través de la capa de peptidoglicano en el caso de *L. monocytogenes*, ya que este es un material de naturaleza porosa que se encuentra en gran proporción en la membrana celular de bacterias Gram positivas, mientras que en los casos de *S.* Enteritidis y *E. coli* O157:H7 entró a través de las porinas, las cuales son proteínas de membrana que forman canales llenos de agua a través de los cuales pueden pasar pequeñas moléculas hidrofílicas, puesto que la estructura de la membrana de estas bacterias es más compleja, presentando una membrana interna y una externa que tiene como función principal actuar como barrera impidiendo el paso de moléculas hidrofilícas dañinas para la célula (Brul y Coote, 1999; Seltmann y Holst, 2002; Nikaido, 2003).

Determinación de las concentraciones mínimas inhibitorias (CMIs) y mínimas bactericidas (CMBs) de aceites esenciales contra *Listeria innocua*, *Salmonella* Enteritidis y *Escherichia coli* en zumos de frutas.

Los aceites esenciales de canela, clavo, hierba de limón, palmarosa y benzaldehido, así como también compuestos activos como eugenol y geraniol fueron seleccionados por su gran actividad antimicrobiana contra microorganismos patógenos reportada en la literatura (Kim y col., 1995; Velluti y col., 2003: Burt, 2004; Oussalah y col., 2007), así como por ser considerados más compatibles con frutas y sus productos. Después de seleccionar los aceites esenciales y compuestos activos a evaluar, se procedió a determinar las CMIs y CMBs contra *L. innocua*, *S.* Enteritidis y *E. coli* en zumos de manzana, pera y melón usando el método de dilución en agar propuesto por Davidson y Parish (1989).

Todos los aceites esenciales y compuestos activos utilizados mostraron actividad antibacteriana contra *L. innocua*, *S.* Enteritidis y *E. coli*, solo que en diferentes grados. Así, hierba de limón, canela, geraniol, clavo y benzaldehido resultaron más efectivos que palmarosa para inhibir el crecimiento de *E. coli*, mientras que el crecimiento de *S.* Enteritidis fue mejor inhibido por hierba de limón, canela, geraniol y palmarosa que por clavo o benzaldehido y el crecimiento de *L. innocua* por hierba de limón, geraniol y palmarosa. A pesar de las diferencias en efectividad una concentración mínima inhibitoria de 1 µl/ml para los tres microorganismos fue encontrada para todos los compuestos evaluados.

Los aceites esenciales de hierba de limón y canela, así como también geraniol fueron escogidos por mostrar mayor efectividad contra los tres microorganismos para el estudio de la determinación de las concentraciones mínimas bactericidas (CMBs) en caldo triptona de soja (TSB) y zumos de manzana, pera y melón usando el método de dilución en caldo propuesto por Davidson y Parish (1989). Los resultados fueron analizados individualmente para cada microorganismo, demostrando la existencia de diferencias significativas ($p < 0.05$) entre las sustancias evaluadas, sus concentraciones y el medio de crecimiento. Así, mayores concentraciones de aceites esenciales fueron requeridas para inactivar las poblaciones de cada microorganismo en zumo de melón y medio TSB que en zumo de manzana y pera. En tal sentido, Nychas y col. (2003) reportaron que la actividad bactericida de los compuestos antimicrobianos naturalmente presentes en plantas es influenciada por el medio de cultivo, la temperatura de incubación y el tamaño del inóculo. Una concentración mínima bactericida (CMB) de 2 µl/ml de los aceites esenciales de hierba de limón y canela así como también de geraniol fue encontrada para los tres microorganismos en zumos de manzana y pera, sin embargo en zumo de melón y medio TSB mayores concentraciones de estas sustancias fueron requeridas.

El aceite esencial de canela y el geraniol demostraron ser más efectivos contra *S.* Enteritidis en zumo de melón y medio TSB que el aceite esencial de hierba de limón ya que una concentración de 2 µl/ml de los primeros fue suficiente para inactivar el microorganismo en estos medios, mientras que una concentración de 5 µl/ml de hierba de limón fue necesaria para lograr el mismo efecto. A diferencia de estos resultados, 2 µl/ml de aceite de hierba de limón o aceite de canela fueron suficientes para eliminar la población de *E. coli* en zumo de melón y medio TSB, mientras que 6 µl/ml de geraniol fueron requeridos para lograr tal inactivación. Concentraciones de 2 µl/ml de aceite de hierba de limón y 6 µl/ml de geraniol fueron también efectivas para inactivar la población de *L. innocua* en zumo de melón y medio TSB, mientras que de aceite de canela mayores concentraciones fueron necesarias (8 µl/ml en zumo de melón y 10 µl/ml en medio TSB). En general, el aceite de hierba de limón demostró ser más efectivo que el aceite de canela y geraniol en zumo de melón y TSB para los tres microorganismos, puesto que una concentración de 5 µl/ml fue suficiente para eliminarlos a los tres, mientras que mayores concentraciones de aceite de canela (10 µl/ml) o geraniol (6 µl/ml) fueron requeridas para lograr el mismo efecto.

Los estudios de microscopía de barrido confocal (CSLM) realizados a células de *S.* Enteritidis cultivadas en medio TSB y zumo de manzana con o sin aceite esencial de hierba de limón permitieron evidenciar el efecto bactericida de este aceite, mientras que los estudios de microscopía electrónica de transmisión (TEM) permitieron observar los daños a nivel celular causados por el mismo. Daños en el contenido citoplasmático de las células de *S.* Enteritidis causados por el propio zumo de manzana sin aceite esencial añadido fueron observados, demostrando que una acción antimicrobiana fue ejercida por las características propias del zumo, como por ejemplo su pH o constituyentes del zumo como el ácido málico. Además, importantes daños de la membrana celular de este microorganismo fueron observados como consecuencia de la adición de aceite esencial de hierba de limón al zumo de manzana. Estos resultados demostraron que el aceite esencial de hierba de limón difundió a través de la membrana celular probablemente favorecido por la composición lipídica de la misma, causando alteraciones en la permeabilidad de la membrana que permitieron inicialmente la salida de iones y otros constituyentes celulares y posteriormente la de macromoléculas indispensables causando así la muerte del microorganismo, como ha sido sugerido por otros investigadores (Burt, 2004).

Aplicación de sustancias antimicrobianas naturales por tratamientos de inmersión para inactivar microorganismos patógenos y extender la vida útil de manzanas y peras frescas cortadas.

La efectividad del ácido D-L-málico (2,5% p/v) como agente antimicrobiano en combinación con N-acetil-L-cisteína (1% p/v), glutation (1% p/v) y lactato de calcio (1% p/v) como sustancias estabilizadoras de color y firmeza para inactivar microorganismos patógenos como *L. monocytogenes*, *S.* Enteritidis y *E. coli* O157:H7 inoculados sobre manzanas y peras frescas cortadas y extender la vida útil de estas frutas frescas cortadas desde un punto de vista microbiológico y fisicoquímico fue evaluada.

La compatibilidad encontrada entre las características sensoriales de manzanas y peras (entre ellas principalmente su acidez) y el uso de ácido málico como agente antimicrobiano en pruebas preliminares realizadas en nuestro laboratorio nos condujeron a seleccionar estas frutas para la realización de este estudio. Por otra parte, las concentraciones utilizadas de

sustancias estabilizadoras de firmeza y color fueron basadas en estudios previos reportados en la literatura (Gorny y col., 2002; Oms-Oliu y col., 2006; Rojas-Graü y col., 2006), mientras que para inactivar los microorganismos patógenos intencionalmente inoculados e inhibir el crecimiento de la flora nativa de las frutas frescas cortadas la concentración de ácido málico utilizada fue seleccionada basado en los resultados previamente obtenidos en zumos de frutas.

Inactivación de *L. monocytogenes*, *S.* Enteritidis y *E. coli* O157:H7

La actividad antimicrobiana del ácido málico en combinación con sustancias estabilizadoras de la calidad contra *L. monocytogenes*, *S.* Enteritidis y *E. coli* O157:H7 intencionalmente inoculadas en un nivel aproximado de 10^6 UFC/g en manzanas y peras frescas cortadas fue evaluado a través de tratamientos de inmersión en soluciones conteniendo N-acetil-L-cisteína, glutation y lactato de calcio (CGLA) y N-acetil-L-cisteína, glutation, lactato de calcio y ácido málico (CGLA + AM). Un tratamiento de estas frutas cortadas en agua destilada (A) fue también incluido como referencia o control.

Los recuentos de *L. monocytogenes*, *S.* Enteritidis y *E. coli* O157:H7 fueron significativamente ($p < 0.05$) afectados por la condición de tratamiento de las manzanas y peras frescas cortadas desde el mismo día de la preparación de las muestras (t = 0 días). Así, manzanas y peras frescas cortadas inmersas en CGLA o A y luego individualmente inoculadas con estos tres microorganismos no mostraron importantes diferencias en los recuentos de las poblaciones iniciales después del procesamiento, sin embargo aquellas manzanas y peras cortadas tratadas con CGLA + AM exhibieron importantes reducciones en las poblaciones de *L. monocytogenes* y *S.* Enteritidis (> 5 Log_{10} UFC/g), mientras que menores reducciones fueron mostradas por la población de *E. coli* O157:H7 (2,72 Log_{10} UFC/g en manzanas y 2,62 Log_{10} UFC/g en peras) para ese momento (t = 0 días), demostrando así una mayor ácido tolerancia. Derrickson-Tharrington y col. (2005) también reportaron una ácido tolerancia de *E. coli* O157:H7 en manzanas cortadas tratadas con soluciones conteniendo ácido ascórbico al 2.8%, ácido cítrico al 1.7% o zumo de limón comercial con o sin preservantes añadidos, indicando que reducciones entre 0.9-1.3 Log_{10} UFC/g de la población de *E. coli* O157:H7 fueron encontradas cuando esas soluciones fueron utilizadas.

Es importante señalar que colonias características de *L. monocytogenes* y *S.* Enteritidis no fueron detectadas en aquellas muestras

de manzanas y peras frescas cortadas tratadas con CGLA + AM después de un procedimiento de recuperación en agua peptonada tamponada por 20 minutos a 35-37ºC, indicando que una inactivación total de esos microorganismos ocurrió desde el mismo día de la preparación de las muestras.

Comportamientos distintos durante el almacenamiento a 5ºC de manzanas y peras frescas cortadas fueron observados en las poblaciones de *L. monocytogenes*, *S.* Enteritidis y *E. coli* O157:H7. En tal sentido, las poblaciones de *S.* Enteritidis y *E. coli* O157:H7 mostraron reducciones a lo largo del período de almacenamiento tanto en manzanas como en peras tratadas con CGLA o A como consecuencia de las sustancias añadidas y de la temperatura de almacenamiento o competencia con la microflora nativa de la fruta, respectivamente, mientras que en aquellas manzanas y peras tratadas con CGLA + AM *S.* Enteritidis no fue detectada desde el inicio del período de almacenamiento (t = 0 días) y la población de *E. coli* O157:H7 se redujo hasta niveles indetectables (aun después de un proceso de recuperación) a partir de 3 días en manzanas y 14 días en peras. Por el contrario, la población de *L. monocytogenes* se mantuvo casi constante en manzanas tratadas con A y se redujo en aquellas manzanas tratadas con CGLA como resultado de la acción antimicrobiana de las sustancias estabilizadoras, mientras que en peras cortadas su población incrementó, confirmando su condición psicrotrófica (Lou y Yousef, 1999). Estos resultados están en concordancia con los reportados por Lanciotti y col. (2003) quienes indicaron que *L. monocytogenes* sobrevivió pero no se multiplicó en manzanas frescas cortadas, así como también con los resultados de Corbo y col. (2005) quienes demostraron que *L. monocytogenes* creció en peras frescas cortadas almacenadas a 4ºC.

Un valor de pH inferior a 4.3 encontrado en manzanas frescas cortadas tratadas con las diferentes soluciones de inmersión incluyendo agua destilada podría justificar porque no ocurrió crecimiento de las poblaciones de *L. monocytogenes* y *S.* Enteritidis a lo largo del tiempo de almacenamiento, ya que el valor mínimo de pH reportado para el crecimiento de estos microorganismos en la literatura es 4,3 (Lou y Yousef, 1999; Jay y col., 2005), mientras que las peras frescas cortadas al presentar un valor de pH mayor a este mínimo de crecimiento permitieron el crecimiento de *L. monocytogenes*, la cual se vio favorecida por su condición psicrotrófica. Sin embargo las razones que podrían justificar porque *E. coli* O157:H7 en manzanas y peras cortadas y *S.* Enteritidis en peras no crecieron podrían ser la temperatura de almacenamiento (5ºC) y la

competencia con la flora nativa, las cuales influyeron significativamente en su supervivencia y crecimiento.

Extensión de la vida útil de manzanas y peras frescas cortadas

Cambios microbiológicos

El comportamiento de microorganismos mesófilos, psicrófilos así como también el de mohos y levaduras naturalmente presentes en manzanas y peras frescas cortadas tratadas con soluciones de CGLA, CGLA + AM o A y almacenadas a 5ºC fue evaluado durante 30 días de almacenamiento con la finalidad de conocer la estabilidad microbiológica en el tiempo de este tipo de productos.

Los recuentos de cada población obtenidos para cada fruta fueron modelados utilizando la ecuación de Gompertz modificada por Zwietering y col. (1990) con el objetivo de visualizar en una forma matemática el efecto de las diferentes sustancias naturales añadidas durante el procesamiento.

Los resultados demostraron que un crecimiento significativo ($p < 0.05$) de las diferentes poblaciones nativas ocurrió en manzanas y peras frescas cortadas durante el tiempo de almacenamiento independientemente de la condición de inmersión utilizada. Por otra parte, se pudo observar también que la condición de inmersión ejerció un efecto significativo ($p < 0.05$) sobre los recuentos de esas poblaciones, mostrando las manzanas y peras frescas cortadas tratadas con una solución conteniendo CGLA + AM menores recuentos de microorganismos mesófilos, psicrófilos y mohos y levaduras que las tratadas con una solución conteniendo CGLA o aquellas tratadas con A.

Los parámetros de crecimiento calculados a partir de la ecuación de Gompertz permitieron interpretar fácilmente los efectos de las diferentes sustancias añadidas sobre el crecimiento de la flora nativa de las manzanas y peras frescas cortadas. De esta manera se pudo observar que el crecimiento de microorganismos mesófilos, psicrófilos, así como también el de mohos y levaduras fue retardado por la acción antimicrobiana ejercida por el ácido málico y las sustancias estabilizadoras de firmeza y color añadidas a las frutas cortadas a través de los tratamientos de inmersión, ya que un menor crecimiento alcanzado en la fase estacionaria (A) y una menor tasa de crecimiento (μ_{max}) así como también tiempos de latencia (λ)

más prolongados fueron encontrados en aquellas manzanas y peras frescas cortadas inmersas en soluciones con esas sustancias incorporadas, siendo intensificado este efecto con la incorporación del ácido málico en la solución de inmersión. Resultados similares a los encontrados en el presente estudio demostrando acción antimicrobiana por parte de ácidos orgánicos aplicados a frutas mínimamente procesadas, así como también por sustancias usadas como estabilizadoras de la calidad en alimentos han sido reportados en la literatura. Así, Pao y Petracek (1997) lograron retardar el crecimiento de la flora nativa en naranjas peladas usando ácido cítrico en diferentes concentraciones, mientras que Jay y col. (2005) indicaron que agentes antioxidantes como el butilhidroxianisol (BHA), butilhidroxitolueno (BHT), ácido etilendiaminotetraacético (EDTA), citrato de sodio, ácido laúrico, etc, han demostrado tener actividad antimicrobiana contra un amplio rango de microorganismos, incluyendo algunos virus y protozoarios.

La ecuación de Gompertz modificada por Zwietering y col. (1990) también fue utilizada en este trabajo para calcular la vida útil desde un punto de vista microbiológico de manzanas y peras frescas cortadas inmersas en diferentes soluciones, tomando en cuenta la regulación Española para el procesamiento, distribución y comercio de comidas preparadas (B.O.E., 2001), la cual indica que el límite máximo permitido de microorganismos aerobios mesófilos para la fecha de caducidad o expiración del producto es 10^7 CFU/g. Así mismo, la vida útil fue calculada para las poblaciones de microorganisms psicrófilos y mohos y levaduras dada la importancia que pueden tener para la fruta fresca cortada almacenada a temperaturas de refrigeración. Los resultados demostraron que en general una extensión de la vida útil de manzanas y peras frescas cortadas fue lograda con el uso de tratamientos de inmersión en soluciones conteniendo sustancias estabilizadoras y ácido málico. Por otra parte, hay que señalar que la vida útil de manzanas frescas cortadas fue limitada por los microorganismos mesófilos (incluyendo bacterias y hongos) independientemente de la solución de inmersión utilizada, mientras que en peras frescas cortadas la flora limitante dependió de la condición de inmersión, siendo la vida útil de peras frescas cortadas inmersas en A limitada por mesófilos incluyendo principalmente bacterias mas que mohos y levaduras, mientras que la vida útil de peras frescas cortadas tratadas con soluciones de inmersión conteniendo CGLA fue limitada principalmente por mohos y levaduras y la de aquellas peras cortadas tratadas con CGLA +MA fue extendida hasta el final del experimento no siendo limitada hasta ese momento por ninguna de las tres poblaciones estudiadas. Estos

resultados demuestran como la condición de tratamiento puede modificar las características de un alimento favoreciendo el crecimiento de una u otra flora.

Cambios fisicoquímicos

Cambios en la composición de gases del espacio de cabeza

Una disminución en la concentración de oxígeno y un aumento en la producción de CO_2, etileno y etanol fueron encontrados en general en manzanas y peras frescas cortadas envasadas bajo aire y almacenadas a 5ºC por 30 días. Estos resultados demostraron que la fruta fresca cortada sigue siendo un tejido vivo que respira y por eso disminuye la concentración de oxigeno y aumenta la de CO_2 en las bandejas, además de que la flora nativa de la fruta se va multiplicando en el tiempo contribuyendo con estos cambios. Asimismo, una disminución de la concentración de oxígeno en las bandejas crea una condición parcialmente anaeróbica que hace que los microorganismos transformen los azúcares de las frutas en etanol y CO_2 contribuyendo al incremento de estos compuestos en el espacio de cabeza de las bandejas. Por otra parte, nuestros resultados confirman que la producción de etileno incrementa en el tiempo debido a que los trozos de manzanas y peras continúan madurándose aun después de ser cortadas las frutas, siguiendo así un clásico comportamiento de frutas climatéricas (Wills y col., 1998). Cambios similares en el tiempo en la composición de gases del espacio de cabeza de bandejas de manzanas "Fuji" y peras "Conference" frescas cortadas fueron reportados por Rojas-Graü y col. (2007a) y Soliva-Fortuny y col. (2004).

La condición de tratamiento o de inmersión ejerció un efecto significativo ($p < 0.05$) sobre el consumo de oxígeno y la producción de CO_2 de manzanas y peras frescas cortadas. Un menor consumo de oxígeno a lo largo del tiempo de almacenamiento (30 días) y una menor producción de CO_2 fueron detectadas en manzanas y peras frescas cortadas inmersas en una solución conteniendo N-acetil-L-cisteína (1%), glutation (1%), lactato de calcio (1%) y ácido D-L-málico (2,5%) (CGLA + AM) que en una solución conteniendo las mismas sustancias estabilizadoras sin ácido málico (CGLA) o en agua destilada (A) como tratamiento control. Estos resultados son una consecuencia directa de la acción antimicrobiana del ácido málico sobre la flora nativa de las manzanas y peras frescas cortadas, ya que una menor población microbiana implica un menor consumo de oxígeno y por consiguiente una menor producción de CO_2.

Cambios en firmeza

Manzanas frescas cortadas envasadas bajo aire y almacenadas a 5ºC por 30 días no mostraron cambios significativos ($p > 0,05$) de firmeza por efecto de la condición de inmersión o el tiempo de almacenamiento. En contraste, las peras frescas cortadas tratadas con CGLA (9,903 N) o CGLA + AM (9,165 N) mostraron una mayor firmeza desde el mismo día de la preparación de las muestras que las peras inmersas en agua destilada (7,217 N), demostrando que el lactato de calcio añadido a la solución de inmersión tuvo un importante efecto sobre la firmeza de las peras frescas cortadas. Por otra parte, una disminución significativa ($p < 0,05$) de la firmeza durante el almacenamiento fue observada en peras frescas cortadas inmersas en agua, indicando que una liberación del calcio de las células ocurrió como consecuencia de los daños que sufrió el tejido de la fruta durante su procesamiento, mientras que en las peras frescas cortadas tratadas con CGLA o CGLA + AM esa disminución de firmeza fue prevenida por el lactato de calcio añadido. Al respecto, Poovaiah (1986) reportó que el calcio juega un papel importante en el mantenimiento de la estructura de la pared celular del tejido vegetal, debido a que interactúa con el ácido péctico para formar pectato de calcio ayudando así al mantenimiento de la firmeza del tejido. Cloruro de calcio, lactato de calcio y propionato de calcio han sido previamente utilizados como agentes estabilizadores de la textura en el procesamiento de manzanas, peras y melones frescos cortados con resultados satisfactorios (Ponting y col., 1972; Luna-Guzmán y Barret, 2000; Gorny y col., 2002; Soliva-Fortuny y col., 2002a, 2002b; Alandes y col., 2006; Quiles y col., 2007; Rojas-Graü y col., 2007a), sin embargo algunos investigadores han indicado que las frutas frescas cortadas tratadas con lactato de calcio han mostrado tener mejor sabor que las tratadas con cloruro de calcio (Ponting y col., 1972; Luna-Guzmán y Barret, 2000).

Las diferencias observadas entre manzanas y peras frescas cortadas con respecto al mantenimiento de la firmeza pueden ser debidas al tipo de fruta y variedad, así tenemos que similarmente a nuestros resultados Rojas-Graü y col., 2007a encontraron que manzanas "Fuji" frescas cortadas parcialmente maduras mantuvieron su firmeza durante el almacenamiento, mientras que Soliva-Fortuny y col. (2002a), (2002b) y (2004) encontraron una disminución de la firmeza en manzanas "Golden delicious" y en peras "Conference" frescas cortadas en el tiempo.

Cambios en color

La condición de inmersión y el tiempo de almacenamiento ejercieron un importante efecto sobre el color de las manzanas y peras

frescas cortadas. Así, menores valores de luminosidad (L*) y tono (ángulo Hue, hº) desde el día de la preparación de las muestras fueron observados en manzanas y peras frescas cortadas inmersas en agua (A) que en soluciones conteniendo sustancias estabilizadoras (CGLA, CGLA + AM). Estos resultados indican que un rápido pardeamiento de la fruta fresca cortada tratada con agua destilada ocurrió durante las primeras horas de procesamiento, mientras que en manzanas y peras tratadas con soluciones incluyendo N-acetil-L-cisteína y glutation (CGLA, CGLA + AM) estos cambios bruscos de color fueron prevenidos. Por otra parte una disminución a lo largo del tiempo de almacenamiento de los parámetros de calidad L* y hº fue también observada, siendo significativa ($p < 0.05$) a partir de los 14 días de almacenamiento. Los importantes cambios observados en el color de manzanas y peras frescas cortadas pueden ser consecuencia de los daños causados al tejido de la fruta durante el procesamiento, ocurriendo una descompartamentalización de enzimas y sustratos que permitió la formación de compuestos coloreados que causaron el pardeamiento de la fruta fresca cortada como sugirieron Varoquaux y Wiley (1994).

Los resultados de estos estudios demostraron que las sustancias seleccionadas como agentes estabilizadores del color de la fruta fresca cortada (N-acetil-L-cisteína y glutation) resultaron apropiadas ya que lograron mantener los parámetros de color de las manzanas y peras frescas cortadas sin cambios significativos por al menos 14 días. Similares resultados han sido reportados por otros investigadores (Molnar-Perl y Friedman, 1990; Rojas-Graü y col., 2006 y 2007a; Oms-Oliu y col., 2006). En tal sentido, Molnar-Perl y Friedman (1990) y Richard y col. (1991) y Richard-Forget y col. (1992) indicaron que cisteína, N-acetil-L-cisteína y glutation reducido pueden prevenir el pardeamiento de la fruta por una reacción competitiva con la enzima polifenol oxidasa, reaccionando con quinonas intermediarias para formar compuestos estables incoloros.

Cambios en las características sensoriales

Cambios significativos en las características de manzanas y peras frescas cortadas debidos a la acción del tiempo de almacenamiento no fueron detectados por los panelistas, sin embargo diferencias significativas entre manzanas y peras frescas cortadas dependientes de las condiciones de inmersión si fueron encontradas. Así, una mejor aceptación de manzanas y peras frescas cortadas tratadas con CGLA y CGLA + AM fue encontrada en comparación con éstas tratadas con agua destilada como control, principalmente debido a su color, sabor y acidez.

Finalmente de todos nuestros resultados podemos decir que una combinación de ácido málico con N-acetil-L-cisteína, glutation y lactato de calcio resultó apropiada para garantizar la inocuidad de manzanas y peras frescas cortadas basada en la inactivación de microorganismos patógenos como *L. monocytogenes*, *S.* Enteritidis y *E. coli* O157:H7, así como también para extender la vida útil de estas frutas frescas cortadas desde un punto de vista microbiológico y fisicoquímico.

Uso de recubrimientos comestibles a base de alginato conteniendo sustancias antimicrobianas naturales para inactivar microorganismos patógenos y extender la vida útil de manzana y melón frescos cortados.

Manzanas y melones fueron seleccionados para evaluar la efectividad de la incorporación de compuestos antimicrobianos a través de recubrimientos comestibles por ser las frutas principalmente involucradas en brotes de enfermedades transmitidos por frutas frescas cortadas y zumos de frutas en los últimos años (Harris y col., 2003). De la misma manera *E. coli* O157:H7 y *Salmonella* fueron escogidos como microorganismos patógenos para estos estudios debido a su relación con los brotes ocurridos por consumo de derivados de manzana (zumos y sidra) y melón fresco cortado, respectivamente.

Los aceites esenciales de canela, hierba de limón, clavo y palmarosa así como también sus principales compuestos activos cinamaldehido, citral, eugenol y geraniol fueron seleccionados basado en su efectividad antimicrobiana contra microorganismos patógenos demostrada en la primera parte de esta investigación. Concentraciones de 0,3 y 0,7% de estos aceites esenciales y de 0,5% de sus compuestos activos fueron individualmente incorporadas en una solución a base de alginato al 2% y glicerol al 1,5% para formar el recubrimiento comestible. Además una solución conteniendo N-acetíl-L-cisteína (1%), glutation (1%), ácido málico (2,5%) y lactato de calcio (2%) para el caso de manzana fresca cortada y ácido málico (2,5%) y lactato de calcio (2%) para melón fresco cortado fue utilizada para facilitar la formación del recubrimiento comestible sobre la superficie de la fruta fresca cortada. Un recubrimiento comestible sin EOs o compuestos activos conteniendo únicamente compuestos estabilizadores de la calidad y ácido málico (EC) fue también evaluado como control. Es importante señalar que las sustancias

estabilizadoras de calidad utilizadas y sus concentraciones fueron seleccionadas en base a estudios previos realizados en el mismo laboratorio (Rojas-Graü y col., 2006; Oms-Oliu y col., 2006), mientras que las concentraciones de EOs y ácido málico fueron seleccionadas basado en los primeros experimentos de esta investigación.

Inactivación de *E. coli* O157:H7 y *S.* Enteritidis en manzanas y melones frescos cortados.

Un recubrimiento a base de alginato con ácido málico y compuestos estabilizadores de color y firmeza sin aceites esenciales incluidos (EC) resultó efectivo para reducir la población de *E. coli* O157:H7 inoculada (10^6 UFC/g) sobre trozos de manzana observándose una disminución de su población de 1,23 ciclos log justo después del procesamiento. Sin embargo una mayor reducción de su población fue alcanzada cuando EOs o sus compuestos activos fueron incorporados al recubrimiento, intensificándose el efecto cuando altas concentraciones (0,7%) de estos compuestos fueron usadas. Además la efectividad de los EOs y sus compuestos activos varió mostrando el aceite esencial de hierba de limón y el citral la mayor actividad antimicrobiana, alcanzándose reducciones de 4,02 y 3,61 ciclos log el mismo día de la preparación de las muestras. Por otra parte, se observaron disminuciones de *E. coli* O157:H7 a lo largo del tiempo, llegando su población a niveles indetectables a partir de los 3 días de almacenamiento en aquellos trozos de manzana recubiertos con soluciones que incluían EOs de hierba de limón y canela así como también sus compuestos activos citral y cinamaldehido.

Similares resultados fueron observados para la población de *S.* Enteritidis inoculada (10^6 UFC/g) en trozos de melón, donde una reducción de 1,35 ciclos log fue alcanzada con el uso de EC, mientras que con el uso de recubrimientos comestibles conteniendo EOs o sus compuestos activos se observaron reducciones mayores que variaron entre 1,41 y 2,13 ciclos log, dependiendo del tipo de EO o compuesto activo usado y de su concentración, resultando el aceite esencial de hierba de limón el más efectivo. Reducciones adicionales de la población de *S.* Enteritidis fueron además observadas durante el almacenamiento.

Un efecto antimicrobiano intensificado por la combinación de ácido málico y los EOs o compuestos activos sobre *E. coli* O157:H7 y *S.* Enteritidis explican las importantes reducciones observadas en las poblaciones de estos patógenos más que las condiciones de anaerobiosis que pueden crear los recubrimientos comestibles aplicados a frutas ya que

es bien conocido que estos dos microorganismos son anaerobios facultativos (Jay y col., 2005).

Las diferencias encontradas en cuanto a la efectividad de los EOs usados en bajas (0,3% v/v) o altas concentraciones (0,7% v/v) pueden ser explicadas por los daños celulares que pueden causar, siendo más significativos cuando las concentraciones usadas son mayores. En tal sentido Nychas y col. (2003) indicaron que el modo de acción de los EOs depende de su concentración, inhibiendo las bajas concentraciones de ellos las enzimas asociadas con la producción de energía, mientras que las altas concentraciones pueden causar la precipitación de proteínas, siendo en consecuencia más efectivas para inactivar los microorganismos.

Las propiedades fisicoquímicas de los EOs y sus compuestos activos así como el mecanismo de acción de cada uno de ellos permitieron explicar las diferencias en efectividad observadas entre ellos. Así un mayor coeficiente de partición reportado en la literatura para citral (2,8-3,0 Log Pow; OECD-SIDS, 2001), el cual es el compuesto activo principal del aceite esencial de hierba de limón, así como también su influencia sobre la permeabilidad de la membrana celular, produciendo inicialmente la salida de iones y pequeñas moléculas y posteriormente la salida de las macromoléculas causando finalmente una disrupción de la membrana con salida del contenido citoplasmático permitieron justificar el mayor efecto antimicrobiano mostrado por este aceite esencial contra *E. coli* O157:H7 y *S.* Enteritidis en comparación con los otros usados. En tal sentido, Burt (2004) indicó que la hidrofobicidad (la cual está determinada por el coeficiente de partición Log Pow) es una característica muy importante de los EOs ya que es la que determina su entrada a la célula a través de la membrana celular. Además los resultados obtenidos en la primera parte de esta tesis demostraron que una disrupción de la membrana celular era observada en células de *S.* Enteritidis cultivadas en zumo de manzana conteniendo aceite esencial de hierba de limón.

El pH inicial de las frutas influyó notablemente en las reducciones encontradas en las poblaciones de *E. coli* O157:H7 y *S.* Enteritidis. Así, mayores reducciones de *E. coli* O157:H7 en trozos de manzana fueron encontradas en comparación con las reducciones halladas para *S.* Enteritidis en trozos de melón aún utilizando el mismo aceite esencial (hierba de limón), el cual resultó ser el más efectivo en ambos estudios. Esto demuestra que la efectividad de los antimicrobianos puede verse incrementada en alimentos ácidos. En tal sentido Burt (2004) reportó que generalmente la susceptibilidad de las bacterias a los efectos

antimicrobianos de los EOs parece aumentar cuando disminuye el pH del alimento.

Es importante señalar que reducciones de las poblaciones de *E. coli* O157:H7 y *S.* Enteritidis también ocurrieron a lo largo del tiempo de almacenamiento en trozos de manzana y melón no recubiertos, indicando que otros factores tales como la temperatura de almacenamiento (5ºC) y la competencia con la flora nativa de las frutas frescas cortadas pudieron haber influido sobre su supervivencia.

Extensión de la vida útil de manzanas y melones frescos cortados

Cambios microbiológicos

EC resultó ser además efectivo para reducir inicialmente la flora nativa de trozos de manzana y melón así como también para inhibir su crecimiento a lo largo del tiempo de almacenamiento. En tal sentido, tasas de crecimiento ($\mu_{máx}$) más bajas y períodos de latencia (λ) más largos fueron observados para microorganismos mesófilos, psicrófilos y mohos y levaduras en trozos de manzana y melón recubiertos con EC en comparación con aquellos no recubiertos (control). En contraste con estos resultados Rojas-Graü y col. (2007b) reportaron que reducciones de la flora inicial de manzanas frescas cortadas no fueron observadas cuando un recubrimiento a base de puré de manzana-alginato sin aceites esenciales añadidos fue aplicado. Estas diferencias entre resultados se deben principalmente a la acción bactericida del ácido málico añadido en este estudio (EC), el cual causó la reducción inicial de la flora nativa en trozos de manzana y melón.

Por otra parte, una mayor inhibición de la flora nativa fue encontrada cuando EOs o sus compuestos activos fueron incorporados en los recubrimientos, sobre todo cuando altas concentraciones de esos aceites fueron usadas. Una extensión de la vida útil microbiológica de trozos de manzana y melón recubiertos fue por consiguiente alcanzada en comparación con aquellos no recubiertos (control), prolongándose significativamente su vida útil cuando altas concentraciones de EOs o sus compuestos activos fueron añadidos a los recubrimientos comestibles. Un efecto similar fue reportado por Rojas-Graü y col. (2007b) quienes encontraron que aceites esenciales de hierba de limón (1 y 1,5% v/v) y orégano (0,5% v/v) inhibieron el crecimiento de microorganismos

psicrófilos así como también el de mohos y levaduras a lo largo del tiempo de almacenamiento.

Este comportamiento de la flora nativa podría ser explicado por el conjunto de efectos causados por una atmósfera ligeramente anaeróbica creada por el recubrimiento comestible aplicado sobre la superficie de la fruta junto con el importante efecto antimicrobiano logrado por la combinación de ácido málico y EOs o sus compuestos activos, el cual consideramos como más influyente debido a que importantes reducciones de la flora nativa ocurrieron justo después del procesamiento de las frutas (t= 0 días).

Cambios fisicoquímicos

Cambios en la composición de gases del espacio de cabeza

Un menor consumo de O_2 y una menor producción de CO_2 y etanol fueron observados en trozos de manzana y melón recubiertos con EC, sin embargo cuando EOs o sus compuestos activos fueron añadidos a los recubrimientos este efecto fue más significativo. Estos resultados son una consecuencia directa de la reducción inicial observada de la flora nativa así como también de la inhibición de su crecimiento en el tiempo causados por el ácido málico y los EOs añadidos a los recubrimientos. Resultados similares fueron reportados por Rojas-Graü y col. (2007b) quienes indicaron que un menor consumo de O_2 y una menor producción de CO_2 fue observada en trozos de manzanas recubiertos con una cobertura a base de puré de manzana-alginato conteniendo aceites esenciales de hierba de limón (1 y 1,5% v/v) y orégano (0,5% v/v).

Cambios en firmeza

El uso de un recubrimiento comestible sin EOs (EC) favoreció el mantenimiento de la firmeza de trozos de manzana y melón a lo largo del tiempo de almacenamiento como consecuencia de la incorporación de lactato calcio como fue también reportado por Lee y col. (2003) y Rojas-Graü y col. (2007b) en trozos de manzana con recubrimientos comestibles. Sin embargo la adición de EOs o sus compuestos activos afectó significativamente la firmeza de los trozos de frutas desde el mismo día de la preparación de las muestras (t = 0 días), siendo los más influyentes el aceite esencial de hierba de limón y su compuesto activo citral. Estos cambios de firmeza podrían ser explicados por importantes daños estructurales ocurridos en la pared celular del tejido de los trozos de manzana y melón, los cuales provocaron la salida de enzimas que degradan

la pectina produciendo un ablandamiento de los trozos de fruta. De igual manera Rojas-Graü y col. (2007b) observaron una drástica reducción en la firmeza de trozos de manzana cuando aceites de hierba de limón (1,5%v/v) y orégano (0,5%v/v) fueron añadidos a recubrimientos a base de puré de manzana-alginato.

Cambios en color

Diferencias significativas en el color de trozos de manzanas y melones fueron observadas entre tratamientos, mostrando los trozos de frutas no recubiertos importantes cambios seguidos por aquellos trozos tratados con recubrimientos que incluían EOs en altas concentraciones y finalmente aquellos recubiertos con EC o EC más EOs en bajas concentraciones los cuales mantuvieron mejor su color. Los cambios observados en trozos de manzana no recubiertos son principalmente debidos a la acción de las enzimas polifenol oxidasa (PPO) y peroxidasa (POD) las cuales quedan expuestas al oxígeno durante el cortado de la fruta y oxidan compuestos fenólicos naturalmente presentes en la manzana. Estos cambios fueron prevenidos en gran medida en piezas de manzana recubiertas con EC y EC + EOs en bajas concentraciones, gracias a la acción de la N-acetíl-L-cisteína y el glutation añadidos a los recubrimientos, los cuales compiten con las enzimas PPO y POD por las quinonas intermediarias y forman compuestos estables incoloros. Sin embargo, cuando altas concentraciones de EOs fueron añadidas a los recubrimientos esta prevención del oscurecimiento fue afectada, debido posiblemente a que importantes daños en el tejido de la fruta ocurrieron por acción de esos EOs liberándose enzimas y sustratos que reaccionaron produciendo compuestos coloreados. Por otra parte para el caso de los trozos de melón se observó una disminución del índice de blancura y croma durante el tiempo de almacenamiento, haciéndose más significativa en aquellos trozos de melón tratados con altas concentraciones de EOs limitando su vida útil de una manera importante.

Cambios en las características sensoriales

Las manzanas y melones frescos cortados recubiertos con EC mostraron una buena aceptación por los panelistas tanto el mismo día de la preparación de las muestras como después de un período de almacenamiento de 15 y 8 días para manzana y melón, respectivamente. Esto demuestra que el recubrimiento comestible incluyendo ácido málico logró conservar los atributos sensoriales de la frutas a lo largo del

almacenamiento. Estos resultados están en concordancia con los reportados por Debeaufort y col. (1998) quienes indicaron que el uso de recubrimientos comestibles puede reducir las pérdidas de compuestos volátiles de los alimentos previniendo así la pérdida parcial o total del sabor y olor, manteniendo de esta manera su calidad.

Los trozos de melón fresco recubiertos o no con EC fueron igualmente aceptados por los panelistas, sin embargo los trozos de manzana recubiertos con EC fueron preferidos antes que aquellos no recubiertos. Sin embargo la incorporación de EOs en el recubrimiento influyó significativamente sobre las características sensoriales de los trozos de fruta, resultando mejor aceptados los trozos de manzana tratados con aceite esencial de hierba de limón, mientras que para trozos de melón los preferidos fueron aquellos tratados con palmarosa.

Comparación de los métodos de aplicación de sustancias antimicrobianas naturales a frutas frescas cortadas.

Los resultados obtenidos en esta tesis permitieron establecer ventajas y desventajas de cada uno de los métodos empleados para aplicar las sustancias antimicrobianas a frutas frescas cortadas. Así encontramos que una aplicación directa de ácido málico en combinación con compuestos estabilizadores de la calidad (N-acetíl-L-cisteína, glutation y lactato de calcio) a través de tratamientos de inmersión resultó más efectiva para reducir o inactivar microorganismos patógenos como *L. monocytogenes* y *S.* Enteritidis en frutas frescas cortadas que cuando se aplicó a través de recubrimientos comestibles, encontrándose reducciones hasta de más de 5 ciclos log el mismo día de la preparación de las muestras (t = 0 días). Estas diferencias pueden ser debidas a una disminución de la efectividad del ácido málico causada por una interacción entre el lactato de calcio y éste ácido durante la formación del recubrimiento comestible como consecuencia de la propiedad quelante del ácido málico, reduciéndose la disponibilidad de ácido málico para actuar contra los microorganismos inoculados sobre la superficie de la fruta. Además la aplicación de compuestos antimicrobianos a través de tratamientos de inmersión resulta más sencilla y económica para el procesamiento de la fruta que la aplicación de recubrimientos comestibles. Sin embargo la aplicación de recubrimientos comestibles tiene las ventajas de disminuir las pérdidas de vapor de agua de la fruta y compuestos volátiles, suavizar el sabor de los compuestos añadidos ya sea como antimicrobianos o como estabilizadores de la calidad y permitir la aplicación de compuestos lipofílicos como los

aceites esenciales los cuales no pueden ser incorporados a través de tratamientos de inmersión en soluciones acuosas.

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CONCLUSIONES

De los resultados obtenidos y de su interpretación se deducen las siguientes conclusiones:

- Los zumos de manzana, pera y melón inhibieron el crecimiento de *L. monocytogenes*, *S.* Enteritidis y *E. coli* O157:H7 durante al menos 5 días de almacenamiento a 5ºC. Sin embargo a 20 y 35ºC una concentración de 0,2% de ácido málico fue necesaria para inhibir el crecimiento de *L. monocytogenes* y *S.* Enteritidis en zumo de melón, mientras que para inhibir el crecimiento de *E. coli* O157:H7 en ese zumo una concentración mayor del mismo ácido (0,4%) fue necesaria. Por otra parte concentraciones de ácido málico más altas fueron efectivas para reducir los tres patógenos en más de 5 cyclos log en zumos de manzana (2%), pera (2,5%) y melón (2,5%), dependiendo la efectividad del tipo de zumo, temperatura y tiempo de almacenamiento así como también del tipo de microorganismo. Observaciones al microscopio (TEM) demostraron que el ácido málico entró a las células de los patógenos generando una aglutinación del contenido citoplasmático sin disrupción de la membrana celular causando la muerte de los microorganismos.

- Los aceites esenciales de canela, clavo, hierba de limón y palmarosa así como también sus compuestos activos fueron efectivos para inhibir el crecimiento y reducir significativamente las poblaciones de microorganismos patógenos o indicadores de éstos en zumos de frutas. Una concentración de 0,2% de canela, hierba de limón o geraniol fue suficiente para inactivarlos totalmente en zumo de manzana y pera. Sin embargo mayores concentraciones fueron necesarias para lograr inactivarlos en zumo de melón. Observaciones al microscopio (CSLM) y (TEM) permitieron comprobar el efecto bactericida y los daños estructurales de la membrana celular de células de *S.* Enteritidis cultivadas en zumo de manzana.

- Una combinación de ácido D-L málico al 2,5% (p/v) con N-acetíl-L-cisteína (1% p/v), glutation (1% p/v) y lactato de calcio (1% p/v) como sustancias estabilizadoras de la calidad aplicada a piezas de manzana y

pera a través de tratamientos de inmersión causó una reducción de más de 5 ciclos log de las poblaciones de *L. monocytogenes* y *S.* Enteritidis justo después del procesamiento de las frutas (t = 0 días), mientras que esa reducción fue alcanzada para la población de *E. coli* O157:H7 después de 3 y 14 días de almacenamiento a 5ºC en esas mismas frutas.

- El uso de ácido málico en combinación con sustancias estabilizadoras de la calidad en piezas de manzana y pera por tratamientos de inmersión inhibió además significativamente el crecimiento de las poblaciones de aerobios mesófilos, psicrófilos y mohos y levaduras durante el almacenamiento a 5ºC, observándose tasas de crecimiento más bajas y fases lag más prolongadas para esas poblaciones cuando esta combinación de sustancias fue usada, lográndose así una extensión de la vida útil microbiológica de la frutas frescas cortadas.

- Un menor consumo de oxígeno y una menor producción de dióxido de carbono, y etanol durante el almacenamiento a 5ºC fue observado en trozos de manzana y pera tratados con una combinación de ácido málico y compuestos estabilizadores de la calidad como resultado de la inhibición del crecimiento de la flora nativa. Además se logró mantener mejor la textura y el color como consecuencia de la incorporación de lactato de calcio y N-acetíl-L-cisteína y glutation, alcanzando en conjunto una extensión de la vida útil fisicoquímica del producto.

- La aplicación de un recubrimiento a base de alginato conteniendo ácido málico como antimicrobiano a piezas de manzana y melón resultó efectiva para reducir las poblaciones de *E. coli* O157:H7 y *S.* Enteritidis inoculadas así como también para inhibir el crecimiento de la flora nativa. Sin embargo la incorporación de EOs o sus principales compuestos activos junto con ácido málico causó un mayor efecto, el cual fue potenciado con el aumento de las concentraciones de EOs.

- El aceite esencial de hierba de limón incorporado en un recubrimiento comestible a base de alginato fue el que mostró mayor efecto antimicrobiano contra *E. coli* O157:H7 y *S.* Enteritidis en trozos de manzana y melón respectivamente, posiblemente debido a su mayor coeficiente de partición el cual facilitó su difusión a través de la membrana celular de esos microorganismos, causando alteraciones significativas en sus células provocando así su muerte.

- Una extensión de la vida útil microbiológica de trozos de manzana y melón fue lograda con el uso de recubrimientos comestibles a base de alginato conteniendo ácido málico y EOs o sus compuestos activos, como resultado de la acción bactericida de estas sustancias sobre la flora nativa, sin embargo algunas características fisicoquímicas como la firmeza y el color así como también sensoriales como el sabor y el olor se vieron afectadas principalmente cuando altas concentraciones de EOs fueron aplicadas.

- En general los resultados obtenidos en esta investigación demuestran que el uso de ácido málico y aceites esenciales o sus compuestos activos como sustancias antimicrobianas puede resultar una buena alternativa de conservación para frutas frescas cortadas.

CONSIDERACIONES FUTURAS:

- Las concentraciones mínimas inhibitorias y mínimas bactericidas de ácido málico y aceites esenciales contra *L. monocytogenes*, *S*. Enteritidis y *E. coli* O157:H7 encontradas en el presente trabajo podrían contribuir a futuros estudios de investigación donde se apliquen tratamientos combinados para zumos y frutas frescas cortadas.

- Debido a las excelentes propiedades sensoriales que pueden ser logradas con el uso de ácido málico en comparación con otras sustancias antimicrobianas se recomienda la realización de nuevos estudios que consideren el uso de éste ácido en combinación con sustancias estabilizadoras de la calidad para otras frutas ácidas frescas cortadas.

- Se sugieren futuros estudios que consideren la incorporación de otras sustancias antimicrobianas así como también estabilizadoras de la calidad y nutricionales en recubrimientos comestibles ya que han demostrado ser excelentes transportadores de estos compuestos a las frutas frescas cortadas.